**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
ON APPEAL FROM THE EXAMINER TO THE BOARD
OF PATENT APPEALS AND INTERFERENCES**

Applicant(s):	**West, et al.**	Customer No.:	**023640**
Serial No.:	**09/935,168**	Confirmation No.:	**9212**
Filed:	**August 21, 2001**	Group Art Unit:	**1644**
Examiner:	**Huynh, Phuong N.**	Attorney Docket:	**002376.1017**
For:	**Tissue Engineering Scaffolds Promoting Matrix Protein Production**		

MAIL STOP APPEAL BRIEF—PATENT
Commissioner for Patents
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AMENDED APPEAL BRIEF

In response to the Notice of Non-Compliant Appeal Brief mailed January 25, 2007, Appalleants/Applicants respectfully submit this Amended Appeal Brief which includes the appropriate headings in the order indicated by 37 C.F.R. § 41.37.

Appellants have appealed to this Board from the decision of the Examiner, contained in a Final Office Action mailed April 20, 2006 finally rejecting Claims 1-5, 7-9, and 24-35. Appellants filed a Notice of Appeal on July 20, 2006 with the statutory fee of $250.00 under 37 C.F.R. § 41.20(b)(2).

Real Party In Interest

The real party in interest for this Application under appeal is Rice University of Houston, Texas. Rice University is the assignee of the inventors' entire interest in the application, as indicated by an assignment recorded on 02/06/2006 from inventors Brenda K. Mann and Jennifer L. West to Rice University, in the Assignment Records of the PTO at Reel 012589, Frame 0373 (5 pages).

Related Appeals and Interferences

To the knowledge of Appellants' counsel, there are no known interferences or judicial proceedings that will directly affect or be directly affected by or have a bearing on the Board's decision regarding this Appeal. We note, however, that this application was the subject of previously filed Appeal No. 2005-1342, which was withdrawn pursuant to 37 C.F.R. § 1.114(d) via a request for continued examination under 37 C.F.R. § 1.114.

Status of Claims

Claims 1-5, 7-9, and 24-35 are pending in the Application and were rejected in the Final Office Action mailed April 20, 2006. Claims 1-5, 7-9, and 24-35 are set forth in the Claims Appendix, along with an indication of the status of those claims. Claims 1-2, 7-8, and 24-35 are presented for appeal. Applicants are not appealing the rejection of claims 3-5 and 9.

Status of Amendments

All amendments submitted by Appellants have been entered by the Examiner.

Summary of Claimed Subject Matter

Independent Claim 1 recites, "[a] method for making a tissue engineering scaffold for inducing formation of extracellular matrix by cells bound to the scaffold (page 3, ll. 5-9) comprising covalently coupling matrix-enhancing molecules to the scaffold (page 7, ll. 6-8) in an effective density to elicit production of extracellular matrix without increasing cellular proliferation (page 1, ll. 4-7; page 4, l. 29-page 5, l. 2; page 7, ll. 12-13), wherein when the matrix-enhancing molecules are TGF-β (page 2, l. 30-page 3, l. 1), the TGF-β is covalently coupled to the matrix by a polymer tether having a molecular weight between 2000 and 6000

(page 7, ll. 3-5) and is in a density between 1 and 100 ng TGF-β/ml (page 7, ll. 16-19) or in a concentration of between about 4 x 10-6 and 4 x 10-3 nmol/ml (page 7, ll. 19-20)."

Independent Claim 24 recites, "[a] method for making a tissue engineering scaffold (page 3, ll. 5-9), the method comprising: providing a scaffold (page 5, ll. 3-18), a polymer tether (page 7, ll. 1-10), and a matrix-enhancing molecule (page 6, ll. 6-29); covalently coupling the polymer tether to the scaffold (page 7, ll. 6-8); and covalently coupling the matrix-enhancing molecule to the scaffold, wherein the matrix-enhancing molecule is present at a concentration sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell (page 1, ll. 4-7; page 3, ll. 5-9; page 4, l. 29-page 5, l. 2; page 7, ll. 12-13)."

Grounds of Rejection to be Reviewed on Appeal

The grounds of rejection for review are:

(1) the rejection of Claims 24-35 under 35 U.S.C. § 112, first paragraph, as allegedly not enabled by the disclosure;

(2) the rejection of Claims 24-35 under 35 U.S.C. § 112, first paragraph, as the disclosure allegedly does not meet the written description requirement;

(3) the rejection of Claims 24, 28, 31, and 35 under 35 U.S.C. § 102(b) as anticipated by U.S. Patent No. 5,162,430 ('430 Patent). (the cited references are provided in the Evidence Appendix.)

(4) the rejection of Claims 1, 2, 7, and 8, and 24-35 under 35 U.S.C. § 103(a) as obvious over the '430 Patent in combination with various references as follows:

- Claims 1-2, and 8[1] over '430 Patent in view of *Dinbergs*;
- Claims 7 and 8[2] over '430 Patent in view of *Dinbergs*[3] and U.S. Pat. No. 5,935,849 ('849 Patent);
- Claim 8 over '430 Patent in view of Dinbergs and WO 94/23740 or WO 96/27657;
- Claims 24-27 and 32-34 over '430 Patent in view of the '849 Patent;
- Claims 27 and 29 over '430 Patent in view of the '849 Patent and *Dinbergs*;
- Claims 24 and 30 over '430 Patent in view of *Scott-Burden*; and

[1] The rejection of claims 4 and 9 over these references is not being appealed.
[2] The rejection of claim 5 over these references is not being appealed.
[3] Claim 3 stands rejected over the '430 Patent in view of *Dinbergs* and *Scott-Burden*, but this rejection is not being appealed.

- Claims 24 and 34 over '430 Patent in view of WO 94/23740 or WO 96/27657.

Applicants are not appealing the rejection of Claims 3-5 and 9, and Applicants do not intend to pursue prosecution of Claims 3-5 and 9.

ARGUMENTS

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ARGUMENTS

Claims 24-35 stand rejected as failing to meet the enablement requirement under 35 U.S.C. § 112, first paragraph. Claims 24-35 stand rejected as failing to meet the written description under 35 U.S.C. § 112, first paragraph. Claims 24, 28, 31, and 35 stand rejected under 35 U.S.C. § 102(b) as anticipated by U.S. Patent No. 5,162,430 ('430 Patent). Claims 1-5, 7-9, and 24-35 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the '430 Patent in combination with various references as described above. For the reasons discussed below, Applicants respectfully submit that these rejections are improper and should be reversed by the Board.

I. Claims 24-35 are enabled under 35 U.S.C. 112, 1st Paragraph

The Examiner rejected claims 24-35 under 35 U.S.C. § 112, 1st paragraph, as allegedly not enabled by the specification. The Examiner has not made out a prima facie case of nonenablement because the description teaches how to make and use the invention in terms which correspond in scope to the claims. *Staehelin v. Secher*, 24 USPQ2d 1513, 1516 (BAPI 1992) ("a specification which teaches how to make and use the invention in terms which correspond in scope to the claims must be taken as complying with the first paragraph of 35 USC 112 unless there is reason to doubt the objective truth of statement relied upon therein for enabling support." (citation omitted)).

A. The enablement rejection is based on a misconstruction of the claim term "matrix-enhancing molecules"

The Examiner has misconstrued the meaning of "matrix-enhancing molecules," and in so doing has incorrectly concluded that independent claim 24 is not enabled.

Although Applicants have not explicitly defined the term "matrix-enhancing molecule" in the specification, the Examiner characterized the term as having been explicitly defined.[4] Moreover, the Examiner's definition is incorrect. The Examiner stated, "The specification defines matrix-enhancing molecules at page 6 as any glycoproteins, any glycoproteins such as elastin, collagen, TGF-β, agniogensin II [sic], insulin-like growth factors, and ascorbic acid."

[4] Neither does the specification explicitly define "scaffold materials," "tethers," nor "cells"; and the Examiner's assertion to the contrary is mistaken. (*Compare* Final Office Action at 4-5, *with* Application at 5, 7-8.)

(Final Office Action at 4.) The Examiner's definition fails to differentiate between a matrix-enhancing molecules and matrix proteins, which are the product of the activity of matrix-enhancing molecules. In contrast to the Examiner's assertion, the specification states:

> Matrix-enhancing molecules ***which promote increased production of ECM*** can be attached to the scaffold material to induce production of matrix proteins, such as glycoproteins, elastin, and collagen, without substantially increasing cell proliferation. These matrix-enhancing molecules ***include*** TGF-β, angiotensin II, insulin-like growth factors, and ascorbic acid.

(Application at 6, ll. 7-11 (emphasis added).) Thus, matrix enhancing molecules include TGF-β, angiotensin II, insulin-like growth factors, and ascorbic acid, i.e., molecules which promote increased production of ECM, and therefore induce production of matrix proteins.

By misconstruing the meaning of "matrix-enhancing molecules," the Examiner incorrectly concluded that independent claim 24 is not enabled:

> The specification does not teach how to make any and all tissue engineering scaffold using any "matrix-enhancing molecule", any scaffold and any polymer tether. ***This is because definition of matrix-enhancing molecule at page 6 of the specification is any glycoproteins.***

(Final Office Action at 3 (emphasis added).) This misconstruction is clear error, which on its own requires reversal of the rejection.

B. Enablement does not require the amino acid sequences of matrix-enhancing molecules

The Examiner has misconstrued the meaning of "matrix-enhancing molecules," and in so doing has required that Applicants provide amino acid sequences that simply do not exist. In the Final Office Action, the Examiner stated, "[t]he specification does not teach how to make all 'matrix-enhancing molecule' for the claimed method without amino acid sequence." (Final Office Action at 3.) The Examiner's statement with regard to the amino acid sequence of matrix-enhancing molecules has no bearing on enablement of the claims. A skilled artisan does not need to know the amino acid sequence of a particular matrix-enhancing molecule to make and use the claimed methods. In fact, matrix-enhancing molecules such as ascorbic acid do not even have an amino acid sequence.

Furthermore, matrix-enhancing molecules are well known and well documented; there is a great deal of publicly available information about matrix-enhancing molecules. The art cited by the Examiner lists many matrix-enhancing molecules. *See, e.g.*, '430 Patent, col. 6, ll. 55-66; '849 Patent, col. 16, Table 1. "The specification need not disclose what is well known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. (citations omitted)." MPEP § 2164.05(a). Accordingly, no specific sequences are needed for someone of ordinary skill in the art to understand how to make and use the invention, and the Examiner's rejection of the claims for lack of enablement on this ground is error.

C. Applicants' specification provides sufficient guidance, including reference to what is known in the art, to enable claims 24-35

In the Final Office Action, the Examiner stated,

> Given the unlimited number of matrix enhancing molecules, there is insufficient guidance as to which matrix enhancing molecules would induce the production of which extracellular matrix by which cell type without increasing cellular proliferation of the attached cells to the scaffold, much less at which particular concentration for the claimed method.

(Final Office Action at 3.) Applicants disagree because "the law does not require a specification to be a blueprint in order to satisfy the requirement for enablement under 35 U.S.C. 112, first paragraph." *Staehelin v. Secher*, 24 U.S.P.Q.2d 1513, 1516 (BPAI 1992). "That is not to say that the specification itself must necessarily describe how to make and use every possible variant of the claimed invention, for the artisan's knowledge of the prior art and routine experimentation can often fill gaps, interpolate between embodiments, and perhaps even extrapolate beyond the disclosed embodiments, depending upon the predictability of the art." *AK Steel Corp. v. Sollac*, 344 F.3d 1234, 1244, 68 U.S.P.Q.2d 1280 (Fed. Cir. 2003). In this case, each element of the claim identified by the examiner represents well-known subject matter that the specification nevertheless explains and exemplifies.

1. Matrix-enhancing molecules are enabled

One source of guidance may be found in Applicants' independent claim 24 itself, which recites that "the matrix-enhancing molecule . . . elicit[s] production of extracellular matrix by a

cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell." *See* MPEP 2164 ("[W]hen the subject matter is not in the specification portion of the application as filed but is in the claims, the limitation in and of itself may enable one skilled in the art to make and use the claim containing the limitation."). Moreover, suitable matrix-enhancing molecules will be recognizable to those of ordinary skill in the art, with the benefit of Applicants' disclosure. *See* MPEP § 2164.05(a) ("The specification need not disclose what is well known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public." (citations omitted)) To aid in such determination, Applicants' disclosure provides examples of four suitable matrix enhancing molecules (*see* Application at 6, ll. 10-11). Thus, there is disclosure of a broad class of well-known molecules, as well as specific examples with four of them. *See In re Wright*, 999 F.2d 1557, 1561, 27 USPQ2d 1510 (Fed. Cir. 1993) ("Nothing more than objective enablement is required, and therefore it is irrelevant whether [a] teaching is provided through broad terminology or illustrative examples.").

There is no real dispute that suitable matrix-enhancing molecules are well known in the art. Indeed, the prior art cited by the Examiner discloses matrix-enhancing molecules:

- The '430 Patent discloses TGF-β, among others. *See, e.g.*,'430 Patent, col. 6, ll. 55-66.
- *Dinbergs* discloses bFGF and TGF-β. *See, e.g.*, Dinbergs, et al., J. Biol. Chem. 271(47): 29822, Abstract (1996).
- The '849 Patent discloses TGF-β, ascorbic acid, among others. *See, e.g.*,'849 Patent, col. 12, ll. 57-62; col. 16, Table 1.
- *Scott-Burden* discloses angiotensin II. *See, e.g.*, Scott-Burden, et al., J. Cardiovasc. Pharmacol. 16 Suppl 4: S36, Abstract (1990).
- WO 94/23740 discloses TGF-β. *See, e.g.*, WO 94/23740 at 4, ll. 34-35.
- WO 96/27657 discloses TGF-β, among others. *See, e.g.*, WO 96/27657 at 10, l. 23-p. 11, l. 2.

2. Extracellular matrix is enabled

Regarding guidance for "which extracellular matrix," such is provided by Applicants' specification, as well as by what well known to those skilled in the art. First, Applicants' specification provides examples of extracellular matrix components. (Application p. 6, ll. 8-9.) And the extracellular matrix has been well characterized, as the art cited by the Examiner shows. *See, e.g.*, *Dinbergs* at 29826-27, Discussion and references cited therein; *Scott-Burden* at S36,

col. 2 and references cited therein; '849 Patent, col. 14, ll. 31-39, col. 15, l. 3-col. 16, l. 46; WO 96/27657 at 11, ll. 11-13. Thus, like the matrix-enhancing molecules that induce their production, extracellular matrix components are both set forth in the specification and well known in the art.

3. Cell types are enabled

Regarding guidance for "which cell type," such is provided by Applicants' specification, as well as by what well known to those skilled in the art. First, Applicants' specification provides examples of various cell types, as well as sources for such cells. (Application p. 7, l. 21-p. 8, l. 9.) And the use of various cell types is well known in the art, as the art cited by the Examiner shows. *See, e.g., Dinbergs* at 29825, col. 1-2 (discussing endothelial cells and smooth muscle cells); '849 Patent, col. 16, Table 1 (listing a number of cell types); WO 96/27657 at 14, ll. 14-27.

4. Proliferation is enabled

Regarding guidance for "without increasing cellular proliferation of the attached cells to the scaffold," such is provided by Applicants' claims and specification, as well as by what is well known to those skilled in the art. First, claim 24 itself provides guidance because it recites that "the matrix-enhancing molecule . . . elicit[s] production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell." *See* MPEP 2164, discussed above, and case law cited therein. Likewise, the specification describes "[m]atrix-enhancing molecules which promote increased production of ECM . . . without substantially increasing cell proliferation." (Application at 6, ll. 7-10.) Further, the specification provides examples of suitable matrix-enhancing molecules: "These matrix-enhancing molecules include TGF-β, angiotensin II, insulin-like growth factors and ascorbic acid." (Application at 6, ll. 10-11.) The TGF-β species is described in detail, (*see* Application at 6, ll. 12-29; *Id.* p. 8, l. 24-p. 15, l. 2), as is the ascorbic acid species, (*see* Application at 15, ll. 3-14). Taken together, the examples of suitable matrix-enhancing molecules, detailed descriptions of these molecules, and express recitation by the claims enable the invention. Nevertheless, guidance is also provided by the knowledge well known in the art, as the art cited by the Examiner shows. *See, e.g., Dinbergs* at 29822-29; *Scott-Burden* at S36-41; '849 Patent, col. 16, Table 1; *Id.* col. 15, ll. 25-28 ("Various growth factors or chemical compounds, including those

discussed supra, may be added to the ECM components to control the growth and differentiation cells."). Indeed, it would beg credulity for the Examiner to assert that "proliferation" is not an enabled claim term at this advanced state of molecular biology.

5. Concentrations are enabled

Regarding guidance for "at which particular concentration for the claimed method," such is provided by Applicants' claims and specification, as well as by what is well known to those skilled in the art. First, the claim language itself provides a functional concentration of the matrix-enhancing molecule that can be determined by routine testing without undue experimentation: "sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell." *See* MPEP 2164. Applicants' specification also describes that "[t]he optimal density [of the matrix-enhancing molecule] will depend on the type of cells to be attached to the scaffold." (Application at 7, ll. 11-20.) Additionally, the specification provides examples of suitable concentrations of specific matrix-enhancing molecules to use with a specific cell type. (Application at 7, ll. 16-20; *Id.* at 13, l. 26- p. 14, l. 7; *Id.* at 14, ll. 15-19; *Id.* at 14, l. 26-p. 15, l. 2; *Id.* at 15, ll. 9-14); *see* MPEP § 2164.06(b) (providing the example of *In re Bundy*, 642 F.2d 430, 434, 433, 209 USPQ 48 (C.C.P.A. 1981), where even though the specification lacked examples of specific dosages, it did teach that the claimed compositions had certain properties and activities similar to related known compositions and therefore was enabled).

The specification exemplifies how a skilled person could determine a particular concentration with reference to TGF-β and ascorbic acid. (*See* Application at 6, ll. 12-25; *Id.* at 8, l. 24-p. 15, l. 14.) Furthermore, Applicants respectfully submit that the determination of a particular concentration of a particular matrix-enhancing molecule does not require testing that is unduly burdensome, in part because it is known within the art the approximate range of concentrations in which various matrix-enhancing molecules are useful in soluble form. This provides a starting point for those of ordinary skill to begin performing a reasonable amount of experimentation to identify an effective density for the matrix-enhancing molecule of interest in tethered form. Even the Examiner has admitted that "it is within the purview of one ordinary skill [sic] in the art to optimize the concentration of matrix-enhancing molecule for the particular cell type attached to the tissue engineering scaffold to elicit production of extracellular matrix

without increasing cellular proliferation of the attached cell." (Final Office Action at 19.) And this is also recognized by the prior art the Examiner cited. *See* WO 96/27657 at 11, ll. 28-30 ("These useful effects can be determined by tethering a selected growth effector molecule and observing the effect on cell growth using growth assays, such as those described in the examples below.").

Furthermore, Applicants' disclosure sets forth exemplary procedures for conducting such experimentation, which further militates against terming such experimentation "undue." (*See* Application at 8, l. 24-p.15 l. 14 (Examples 1-4).) Such reasonable experimentation is permissible under 35 U.S.C. § 112, because enablement does not hinge on whether ***any*** experimentation is necessary, but only on whether any such experimentation may be termed "undue." MPEP §2164.06 ("'The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.'" (quoting *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400 (Fed. Cir. 1988) (citing *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, (C.C.P.A. 1976)). Where, as here, those of ordinary skill in the art typically engage in some degree of experimentation, such experimentation should not be considered undue.

D. Experimentation, if required, would not be "undue"

Applicants have provided sufficient disclosure that those of skill in the art, with the benefit of Applicants' disclosure, will be able to determine, after reasonable experimentation, suitable "matrix-enhancing molecules" and the "concentration" of a particular matrix-enhancing molecule to use to practice the claimed invention. Yet, the Examiner has also stated:

> Further, there is insufficient working example showing that any matrix enhancing molecule is effective for inducing matrix production in all cell type, in turn, would be useful for implantation. The specification does not teach how to predict which matrix-enhancing molecule is effective for inducing matrix production by which cell type.

(Final Office Action, at 3.)

Applicants respectfully disagree because Applicants' specification provides working examples, and to the extent any experimentation is needed, a skilled person would not consider it undue. *Falko-Gunter Falkner v. Inglis*, 448 F.3d 1357, 1365, 79 U.S.P.Q.2d 1001 (Fed. Cir.

2006) (finding claims enabled and quoting the Board's observation that "the mere fact that the experimentation may have been difficult and time consuming does not mandate a conclusion that such experimentation would have been considered to be 'undue' in this art."); *Enzo Biochem, Inc. v. Calgene, Inc.*, 188 F.3d 1362, 1371, 52 U.S.P.Q.2d 1129 (Fed. Cir. 1999 ("[A] 'reasonable' amount of routine experimentation," is allowed but "such experimentation must not be 'undue.'").

Applicants' specification, through the cited examples, also provides a very detailed description of two disparate species of matrix-enhancing molecules, TGF-β and ascorbic acid. As discussed above, these examples describe how to test different matrix-enhancing molecules to determine, among other things, suitable cells and concentrations. The specification provides descriptions of ways to determine if the desired results have been obtained. Such experimentation, as may be performed to identify all concentrations for matrix-enhancing molecules meeting the claim limitations, merely involves trying new molecules using methods that are well-known or described in the specification. One skilled in the art would also expect other matrix-enhancing molecules to be operable based on the data that Applicants have provided for TGF-β and ascorbic acid.

Moreover, the level of skill in the art further contradicts the Examiner's statement regarding whether any experimentation would be termed "undue." *See, e.g.*, '849 Patent, col. 17, ll. 60-63 ("One of ordinary skill can readily screen a cell type to determine its responsiveness to an ECM molecule or to a cellular ECM from a specific source, to determine its effectiveness in controlling cell distribution."); WO 96/27657 at 11, ll. 28-30 ("These useful effects can be determined by tethering a selected growth effector molecule and observing the effect on cell growth using growth assays, such as those described in the examples below.").

E. Conclusion

The Application enables one of skill in the art to make and use Applicants' claimed invention. The Examiner has failed to meet the burden of proving non-enablement, particularly given the teaching of the specification in light of the level of skill in the art, which is established both by the disclosure and the references in the art, ***including the references cited by the Examiner***. As a result, this rejection is in error and Applicants respectfully request reversal and withdrawal of the rejection of claims 24-35.

II. Claims 24-35 satisfy the written description requirement under 35 U.S.C. 112, 1st Paragraph

An application's written description is "presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the Examiner to rebut the presumption," MPEP § 2163.04 (citing *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (CCPA 1971)), and the burden falls on the Examiner to establish a "reasonable basis to challenge the adequacy of the written description." *Id.* (citing *In re Wertheim*, 541 F.2d 257, 262, 191 U.S.P.Q. 90 (C.C.P.A. 1976). The Examiner must present evidence of "why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims." *Id.* Here, the Examiner has contended:

> The specification does not reasonably provide a written description of (1) any matrix-enhancing molecule, any matrix-enhancing molecule such as angiotensin II, insulin like growth factor, and ascorbic at any concentration sufficient to elicit production of (2) any extracellular matrix by (3) any cell attached to any engineering scaffold.

(Final Office Action at 6-5.) Particularly in view of the fact that Applicants' specification describes matrix-enhancing molecules, concentrations sufficient to elicit ECM production, and types of cell that produces the ECM, the Examiner has not adequately explained why Applicants' written description fails to convey to those skilled in the art that, as of the filing date, applicant was in possession of the invention as claimed. The Examiner has not met her initial burden "of presenting evidence by a preponderance of evidence why a person skilled in the art would not recognize" in the present application, a description of the invention as defined by the claims. Consequently, this rejection is also reversible error and Applicants request withdrawal of the rejection of claims 24-35.

Applicants' claims are supported by an adequate written description. "The subject matter of the claim need not be described literally in order for the disclosure to satisfy the description requirement," but instead, a specification that conveys "with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed" satisfies the written description requirement. MPEP § 2163.02. This invention provides the novel combination of matrix-enhancing molecules (a class of molecules that are well-known in the art and thus adequately described by reference to them generically and by function) covalently coupled to a scaffold (a type of molecule that is also well-known in the art) to achieve

the novel result of eliciting ECM production without increasing cellular proliferation. The Examiner's requirement that all matrix-enhancing molecules must be reported in the specification is clear error—such a requirement does not add descriptive substance. In rejecting claims 24-35 for lack of a written description, the Examiner has neglected the fact that "(1) examples are not required to support the adequacy of a written description (2) the written description standard may be met . . . even where actual reduction to practice of an invention is absent; and (3) there is no *per se* rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure." *Falko-Gunter Falkner v. Inglis*, 448 F.3d 1357, 1366, 79 U.S.P.Q.2d 1001 (Fed. Cir. 2006).

A. Applicants have described matrix-enhancing molecules

At the outset, as noted above in connection with the argument rebutting the enablement rejection, Applicants note that the Examiner has misconstrued the term "matrix-enhancing molecule." Under this misconstruction, there is no way the Examiner could have properly evaluated adequacy of the written description of the specification and should be reversed on this basis alone. Nevertheless, Applicants explain below how the written description requirement has been satisfied.

Applicants' specification adequately describes matrix-enhancing molecules. For example, Applicants' specification states that "[m]atrix-enhancing molecules which promote increased production of ECM can be attached to the scaffold material to induce production of matrix proteins . . . without substantially increasing cell proliferation. These matrix-enhancing molecules include TGF-β, angiotensin II, insulin-like growth factors and ascorbic acid." (Application at 6, ll. 7-11.) Applicant's specification also discloses that the concentration for a specific matrix-enhancing molecule "will depend on the type of cell to be attached to the scaffold." (Application at 7, ll. 15-16.) Moreover, Applicant's specification provides exemplary concentrations of matrix-enhancing molecules. For example, the concentration of the matrix-enhancing molecule TGF-β needed to elicit ECM production in auricular chondrocytes is provided. (Application at 7, ll. 18-20.) As another example, the concentration of another specific matrix-enhancing molecule that is sufficient to produce ECM in aortic smooth muscle cells and auricular chondrocytes is also provided. (Application at 15, ll. 4-14.) Furthermore, as originally filed, Applicants' independent claims 1, 10, and 16 specifically recite that the concentration of

the matrix-enhancing molecule is "an effective density to elicit production of extracellular matrix without increasing cellular proliferation." (Application at 16-17, claims 1, 10, 16.)

Applicants further note that the prior art cited by the Examiner shows that suitable matrix-enhancing molecules are well known. *See, e.g.*, '430 Patent, col. 6, ll. 55-66; '849 Patent, col. 12, ll. 57-62; col. 16, Table 1; *Scott-Burden* at S36, Abstract; WO 96/27657 at 10, l. 23-p. 11, l. 2; *see Capon v. Eshhar*, 418 F.3d 1349, 1358, 76 U.S.P.Q.2d 1078 (Fed. Cir. 2005) ("The 'written description' requirement must be applied in the context of the particular invention and the state of the knowledge."). Thus, suitable matrix-enhancing molecules will be recognizable to those of ordinary skill in the art, with the benefit of Applicants' disclosure. *See* MPEP § 2164.05(a) ("The specification need not disclose what is well known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public." (citations omitted)). In short, "martix-enhancing molecules" refers to a known class of molecules of known structure and function. One of ordinary skill in the art would perceive the claimed subject matter as having been possessed by Applicants. The Examiner fails to establish any evidence to conclude otherwise.

B. Applicants have described extracellular matrix

The Examiner has contended, without providing any evidence or explanation, that there is insufficient written description of any extracellular matrix. (Final Office Action at 6.) The failure to provide reasons for the rejection is sufficient ground for reversal. However, Applicants have provided a written description of the production of extracellular matrix in conjunction with the invention.

Extracellular matrix is well-known in the art as evidenced by the references of record. Moreover, Applicants' specification sufficiently describes extracellular matrix (ECM). Applicants' specification states that ECM includes "matrix proteins, such as glycoproteins, elastin, and collagen." (Application at 6, ll. 8-9.) The specification explains that "[i]n order to maintain proper mechanical integrity of the tissue, the cells must generate sufficient extracellular matrix (ECM)." (Application at 1, ll. 17-19.) Furthermore, the specification describes how to evaluate matrix protein production, including the composition of the ECM. (Application 10, ll. 1-26; *Id.* at 12, ll. 1-5.) In addition to the disclosure provided by Applicants' specification, persons skilled in the art recognize that the extracellular matrix is well characterized, as the art cited by

the Examiner shows. *See, e.g.*, *Dinbergs* at 29826-27, Discussion and references cited therein; *Scott-Burden* at S36, col. 2 and references cited therein; '849 Patent, col. 14, ll. 31-39, col. 15, l. 3-col. 16, l. 46; WO 96/27657 at 11, ll. 11-13.

C. Applicants have described cells attached to the scaffold.

The Examiner has contended, without providing any evidence or explanation, that there is insufficient written description of any cell attached to any engineering scaffold. The failure to provide reasons for the rejection is sufficient ground for reversal. However, Applicants have provided a written description of cell attached to any engineering scaffold in conjunction with the invention.

Applicants' specification adequately describes cells attached to the scaffold. Applicants describe a number of suitable cells and cell sources. For example, the specification states that "[p]referred cells for formation of vascular tissue include smooth muscle cells, endothelial cells, and fibroblasts. Preferred cells for formation of connective tissue include chondrocytes, fibroblasts, and other types of cells that differentiate into bone or cartilage." (Application at 8, ll. 6-9.) Furthermore, the use of various cell types is well known in the art, as the art cited by the Examiner shows. *See, e.g.*, *Dinbergs* at 29825, col. 1-2 (discussing endothelial cells and smooth muscle cells); '849 Patent, col. 16, Table 1 (listing a number of cell types); WO 96/27657 at 14, ll. 14-27.

D. Applicants have described scaffolds

Applicants' specification generally describes scaffold materials (Application at 5, ll. 3-18) and formation of scaffolds (Application at 5, l. 19-p. 6, l. 5). The specification also provides detailed descriptions of specific scaffolds (Application at 9, ll. 23-30; *Id* at 11, ll. 7-22).

Furthermore, Applicants' specification states that "[m]atrix-enhancing molecules which promote increased production of ECM can be attached to the scaffold material to induce production of matrix proteins . . . without substantially increasing cell proliferation. These matrix-enhancing molecules include TGF-β, angiotensin II, insulin-like growth factors and ascorbic acid." (Application at 6, ll. 7-11.) Applicants' specification also discloses that the concentration for a specific matrix-enhancing molecule "will depend on the type of cell to be attached to the scaffold." (Application at 7, ll. 15-16.) In connection, the description provides an example of a specific matrix-enhancing molecule, the concentration needed to elicit ECM

production, and the specific type of cell that produces the ECM. (Application at 7, ll. 18-20.) Moreover, Applicants describe other examples of matrix-enhancing molecules (Application at 15, ll. 3-14), concentrations (Application at 14, ll. 8-19), and cells (Application at 13, ll. 16-25; *Id.* at 14, ll. 9-14; *Id.* at 14, ll. 21-25) in the Examples section of the Application.

E. Applicants have described the correlation of structure and function

Throughout Applicants' specification and claims, the relationship between the structure of a matrix-enhancing molecule and its function is described. In short, the matrix-enhancing molecule, a class of molecule both well-known in the art and described generically and by example in the specification, is covalently coupled to the scaffold through a tether. This tethered matrix-enhancing molecule retains the function of the untethered matrix-enhancing molecule, to increase the production of extracellular matrix by cells. This structure-function relationship is expressly recited in the claims. For example, Applicants' independent claim 24 itself recites "covalently coupling the matrix-enhancing molecule to the scaffold, wherein the matrix-enhancing molecule is present at a concentration sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell." In other words, the matrix-enhancing molecule is present, in a new form (tethered) and amount (effective to produce ECM without increasing proliferation), to perform the function it is known to perform. Likewise, Applicants' specification provides that "[m]atrix-enhancing molecules which promote increased production of ECM . . . without substantially increasing cell proliferation." (Application at 6, ll. 7-11.) And Applicants' specification further describes that "[f]or the matrix-enhancing molecules to induce formation of ECM, it is necessary for the molecule to be tethered to the scaffold by a tether." (Application at 7, ll. 2-3.) Applicants' specification also describes that "[t]he optimal density [of the matrix-enhancing molecule] will depend on the type of cells to be attached to the scaffold." (Application at 7, ll. 15-16.) Additionally, Applicants' specification provides examples of suitable concentrations of specific matrix-enhancing molecules to use with a specific cell type, as well as how a skilled person could determine a particular concentration with reference to TGF-β and ascorbic acid. (*See* Application at 7, ll. 16-20; *Id.* at 13, l. 26- p. 14, l. 7; *Id.* at 14, ll. 15-19; *Id.* at 14, l. 26-p. 15, l. 2; *Id.* at 15, ll. 9-14.)

Accordingly, Applicants' claims and specification expressly provide a correlation between the structure of the matrix-enhancing molecule (i.e., a known class of molecule tethered to a scaffold), and function of the matrix-enhancing molecule (i.e., elicit production of extracellular matrix without increasing cellular proliferation). Moreover, Applicants note that suitable matrix-enhancing molecules and their effects on cells are well known in the art, as the art cited by the Examiner shows. *See, e.g.*, '430 Patent, col. 6, ll. 55-66; '849 Patent, col. 16, Table 1; *Id.* , col. 14, ll. 31-39; *Id.* col. 15, ll. 25-28; *Dinbergs* at 29822-29; *Scott-Burden* at S36-41; *see* MPEP § 2163(II)(A)(3)(a)(C)(2) ("[T]he written description requirement may be satisfied through disclosure of function and minimal structure when there is a well-established correlation between structure and function."). Thus, for these reasons alone, Applicants have satisfied the written description requirement.

F. Applicants have described representative species

Applicants have provided examples of suitable matrix-enhancing molecules: "These matrix-enhancing molecules include TGF-β, angiotensin II, insulin-like growth factors and ascorbic acid." (Application at 6, ll. 7-11.) The TGF-β species is described in detail, (*see* Application at 6, ll. 12-29; *Id.* p. 8, l. 24-p. 15, l. 2), as is the ascorbic acid species, (*see* Application at 15, ll. 3-14). In view of the knowledge of skilled persons and the species disclosed, persons of skill in the art would recognize that Applicants were in possession of the necessary common attributes possessed by matrix-enhancing molecules as claimed in Applicants' claims 24-35. *See* MPEP § 2163(II)(A)(3)(a) ("Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces.").

G. Conclusion

Applicants respectfully submit that the above-cited portions of applicants' specification, along with the rest of the application and the knowledge in the art, are sufficient to convey to one skilled in the art that, as of the filing date, applicants were in possession of the invention as claimed. Accordingly, this rejection is in error and should be reversed.

III. The Examiner has improperly construed the scope of claim 24 by ignoring a limitation

In numerous instances in the Office Actions, the Examiner has improperly construed the scope of Applicants' claims, including claim 24. In so doing, the Examiner has improperly failed to recognize certain limitations that are present in the claim, and that are not disclosed by the cited references.

The Examiner appears to perceive Appellants' claims as lacking a requirement that extracellular matrix production is enhanced without increasing cellular proliferation. For example, the Examiner has stated:

> [T]he specific concentration such as ng/ml or nmol/ml of the specific matrix-enhancing molecule is not recited in independent claim 24. Further, the specific type of cell attached to the engineering tissue scaffold that affected by the specific matrix enhancing molecule is not recited in claim 24.

(Final Office Action at 8.) Similar statements are made elsewhere in the Office Action. (*See* Final Office Action at 18-19, 20, 23, 25.) The Examiner's assertion is untenable in light of the express language of claim 24:

> A method for making a tissue engineering scaffold, the method comprising:
>
> providing a scaffold, a polymer tether, and a matrix-enhancing molecule;
>
> covalently coupling the polymer tether to the scaffold; and
>
> covalently coupling the matrix-enhancing molecule to the scaffold, wherein the matrix-enhancing molecule is present at ***a concentration sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell.***

See, e.g., Appeal Brief, Claims Appendix. Therefore, contrary to the Examiner's contention, claim 24 clearly recites a method for making a tissue engineering scaffold wherein extracellular matrix production is enhanced, or increased, without increasing cellular proliferation. *See Abbot Labs v. Baxter Pharm. Prods. Inc.*, 334 F.3d 1274, 67 U.S.P.Q.2d 1191 (Fed. Cir. 2003) (upholding the non-limiting use of the terms "amount sufficient" and "amount effective").

For a reference to anticipates a claim under 35 U.S.C. § 102, the reference must expressly or inherently described each claim limitations. MPEP §2131. Likewise, when an Examiner asserts that a combination of references obviates a claim under 35 U.S.C. § 103, the

combination "must teach or suggest all the claim limitations." MPEP § 2143. To anticipate or obviate the claim, the prior art must teach that extracellular matrix production be elicited without increasing cellular proliferation, because this is a limitation of claim 24. However, the cited art fails to meet this limitation. As explained in more detail below, none of the references describe the claimed method of coupling a matrix-enhancing molecule to a tissue engineering scaffold in an effective density to increase extracellular matrix production without increasing cellular proliferation. By improperly construing the scope of claim 24, the Examiner has improperly rejected claim 24 as anticipated and obviated by art that clearly does not disclose each limitation in the claim.

IV. The '430 Patent fails to anticipate claims 24, 28, 31, and 35.

By improperly construing the scope of claim 24, the Examiner has improperly rejected claims 24, 28, 31, and 35 as anticipated by art that clearly does not disclose, either expressly or inherently, the claimed method of coupling a matrix-enhancing molecule to a tissue engineering scaffold in an effective density to increase extracellular matrix production without increasing cellular proliferation. The Examiner has not shown that the '430 Patent discloses, either expressly or inherently, each and every limitation recited in Applicants' independent claim 24, which is reversible error

The '430 Patent discloses that "biologically active factors to aid in healing or regrowth of normal tissue" may be chemically linked to a collagen-polymer composition. '430 Patent, col. 6, l. 53-col. 7, l. 5. But the '430 Patent does not disclose using biologically active factors at concentrations that elicit production of extracellular matrix without increasing cellular proliferation. Rather, the '430 Patent discloses concentrations sufficient "to stimulate tissue growth to a detectable degree[, and] [t]issue, in this context, includes connective tissue, bone, cartilage, epidermis and dermis, blood, and other tissue." '430 Patent, col. 7, ll. 17-22. Thus, the '430 Patent teaches stimulating cell proliferation and makes no mention of extracellular matrix production. In other words, the '430 Patent teaches ***proliferation***, in contradistinction to the claimed invention. Accordingly, the '430 Patent does not expressly teach the claimed subject matter.

Nor does the '430 Patent inherently teach the claimed subject matter, as Example 6 of the '430 Patent further illustrates. Example 6 uses a matrix-enhancing molecule (TGF-β)

concentration that is ***not*** sufficient "to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell," as the following calculations demonstrate.

To prepare the collagen-polymer-growth factor conjugates, three components were combined:

- 25μL of a solution of TGF-β1 at a concentration of 1 mg/ml;
- 100μL of a solution of dPEG in CH_2Cl_2; and
- 2.5 mL of collagen solution.

The combination of these components results in a total solution volume of 2.625 mL:

$$(25\,\mu L\ TGF\text{-}\beta 1\ solution)+(100\,\mu L\ dPEG/CH_2Cl_2\ solution)+(2500\,\mu L\ collagen\ solution)=2.625\,mL$$

And the amount of TGF-β1 in the solution is 0.025 mg:

$$\left(\frac{25\,\mu L\ TGF\text{-}\beta 1\ solution}{1}\right)\left(\frac{1\ mg\ TGF\text{-}\beta 1}{1\ mL\ TGF\text{-}\beta 1\ solution}\right)\left(\frac{1\ mL}{1000\,\mu L}\right)=0.025\ mg\ TGF\text{-}\beta 1$$

The mixture of the three components was allowed to react and the resulting pellet was collected and washed. After washing, approximately 40% or 50% of the TGF-β1 is retained in the composition, depending on the reaction time. *See* '430 Patent, col. 19, ll. 54-56. Thus, the amount of TGF-β1 retained in the composition at 40% retention is 0.01 mg, and 0.0125 mg at 50% retention.

To estimate the concentration of TGF-β1 in the composition, the amount of TGF-β1 after washing is divided by the total solution volume. As the total solution volume is much greater than the volume of the pellet, this value underestimates the concentration of TGF-β1.

- For 40% retention: $\left(\frac{0.01\ mg\ TGF\text{-}\beta 1}{2.625\ mL}\right)\left(\frac{10^6\ ng}{1\ mg}\right)=3.809\times10^3\ ng/mL$
- For 50% retention: $\left(\frac{0.0125\ mg\ TGF\text{-}\beta 1}{2.625\ mL}\right)\left(\frac{10^6\ ng}{1\ mg}\right)=4.761\times10^3\ ng/mL$

The concentration of TGF-β1 used in the '430 Patent, even when underestimated, is much greater than the amount of TGF-β "sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell." Accordingly, the '430 Patent fails to inherently disclose every limitation of claim 24.

Thus, contrary to the Examiner's statement that all limitations of claims 24, 28, 31, and 35 are disclosed by the '430 Patent, the concentration limitation is not, so the rejection is unsupported by the art and should be withdrawn.

V. The combination of the '430 Patent and *Dinbergs* fails to obviate claims 1, 2, and 8

Claims 1, 2, and 8 are patentable over the combined teaching of the '430 Patent and *Dinbergs* for several reasons. First, there is no reason, suggestion, or motivation to combine the references in the manner required to produce the claimed invention. Second, the Examiner's obviousness rejection is premised on flawed understanding of *Dinbergs*. Third, even if the references are combined in the manner indicated by the Examiner, the resulting method would not include every limitation recited in Applicants' independent claim 1 because no reference teaches covalently coupling TGF-β to the scaffold in an effective density to elicit production of extracellular matrix without increasing cellular proliferation. Thus, the obviousness rejections are all fatally defective, resulting in reversible error.

A. There is no basis to combine the '430 Patent and *Dinbergs*

1. Both the '430 Patent and *Dinbergs* teach away from the claimed invention

The portions of the '430 Patent and *Dinbergs* that would lead away from the claimed invention must be considered. MPEP § 2141.02(VI) (citing *W.L. Gore & Assoc., Inc.*, 721 F.2d 1540, 220 U.S.P.Q. 303 (Fed. Cir. 1983) ("A prior art reference must be considered in its entirety, *i.e.*, as a whole, including portions that would lead away from the claimed invention.")); *In re Hedges,* 783 F.2d 1038, 1041, 228 U.S.P.Q. 685, 687 (Fed. Cir. 1986) ("It is impermissible within the framework of section 103 to pick and choose from any one reference only so much of it as will support a given position, to the exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one of ordinary skill in the art."). When the '430 Patent and *Dinbergs* are fully considered, they teach away from the claimed invention.

The '430 Patent teaches away from the claimed invention because it teaches cell proliferation, ***the very thing that the claimed invention avoids***. The '430 Patent discloses "biologically active factors to aid in healing or regrowth of normal tissue." '430 Patent, col. 6, l. 53-col. 7, l. 5. And the '430 Patent discloses concentrations sufficient "to stimulate tissue growth to a detectable degree." '430 Patent, col. 7, ll. 17-22. Thus, the '430 Patent teaches stimulating

cell proliferation and makes no mention of extracellular matrix production, which teaches away from Applicant's claimed invention. The inclusion of the '430 Patent in the obviousness rejections represents clear error that must be reversed.

Dinbergs teaches away from the claimed invention because it concerns release of soluble growth factors. *Dinbergs* at 29827. In particular, *Dinbergs* compared TGF-β's effect on cell proliferation when released as a bolus or when release was controlled. *Dinbergs* at 29826. This is in direct conflict with claim 1's requirement to covalently couple the matrix-enhancing molecules to the scaffold, which also necessitates reversal.

2. The '430 Patent and *Dinbergs* are incompatible and cannot be combined

Dinbergs teaches that TGF-β weakly ***inhibits*** cellular proliferation. The '430 Patent teaches promotion of cellular proliferation. Because one reference teaches inhibition of cell growth, while the other reference teaches the promotion of cell proliferation, the '430 Patent and *Dinbergs* are incompatible and cannot be combined.

The '430 Patent teaches that "biologically active factors," like TGF-β, can be chemically linked to a collagen-polymer conjugate to "aid in healing or ***regrowth*** of normal tissue." '430 Patent, col. 6, ll. 54-55 (emphasis added). "Compositions of the invention which contain growth factors are particularly suited for sustained administration of factors, as in the case of wound healing promotion. Osteoinductive factors and cofactors (including TGF-β) may be advantageously incorporated into compositions destined for bone replacement, augmentation, and/or defect repair." '430 Patent, col. 12, l. 63-col. 13, l. 1; '430 Patent, 4, ll. 34-41 ("Such tethering of growth factors to collagen molecules provides an effective slow-release drug delivery system.") The growth factors are used at concentrations sufficient to "stimulate tissue growth to a detectable degree." '430 Patent, col. 7, ll. 18-22.

Dinbergs teaches that growth factors, like TGF-β, may be released by various polymer devices to regulate cell growth. *Dinbergs* at 29827. Furthermore, *Dinbergs* teaches that the naturally occurring release rate for a given growth factor should be considered when choosing the rate to deliver growth factors to cells. *See Dinbergs* at 29826. For TGF-β in particular, *Dinbergs* showed that sustained release from a microsphere inhibited proliferation of smooth muscle cells. *Dinbergs* at 29822-3.

In light of these teachings, a combination of the '430 Patent and *Dinbergs* would change the basic principle under which each reference was designed to operate. The '430 Patent was designed for sustained release of growth factors, such as TGF-β, to "stimulate tissue growth to a detectable degree." '430 Patent, col. 7, ll. 18-22; col. 19, ll. 10-56 and Figure 2. But according to *Dinbergs*, the slow release of TGF-β would actually inhibit cellular proliferation, preventing tissue growth to a detectable degree. Therefore, there would be no motivation to combine the teachings of the '430 Patent and *Dinbergs*, much less any teaching from the combined references to elicit production of extracellular matrix without increasing cellular proliferation.

3. The proposed '430 Patent-*Dinbergs* combination has no reasonable expectation of successfully achieving the invention

Not only does the above-described incompatibility of the '430 Patent with *Dinbergs* preclude a suggestion or motivation to combine, but it also signifies that an ordinary artisan would not have reasonably expected that a combination or modification of the references would be successful.

As discussed above, *Dinbergs* teaches that the use of TGF-β in the '430 Patent would not succeed in stimulating tissue growth, because sustained release of TGF-β does a better job of inhibiting proliferation than promoting it. And the '430 Patent teaches that cellular proliferation is what is needed to stimulate tissue growth to a detectable degree. Because the teachings of the '430 Patent and *Dinbergs* are incompatible, a skilled artisan would have no reason to expect that the '430 Patent-*Dinbergs* combination would be successful to elicit production of extracellular matrix without increasing cellular proliferation.

B. The Examiner's obviousness rejection is premised on flawed understanding of *Dinbergs*

The Examiner's motivation to combine the '430 Patent and *Dinbergs* is wrong because it is based on a flawed understanding of *Dinbergs*. The Examiner has mischaracterized what *Dinbergs* discloses. (*See* Final Office Action at 9-10.) Contrary to the Examiner's assertions, *Dinbergs* does not disclose a method of making a tissue engineering scaffold, or that matrix enhancing molecules may be coupled to such a tissue engineering scaffold. Rather, *Dinbergs* teaches that growth factors, like TGF-β, may be released by various polymer devices to regulate cell growth. *Dinbergs* at 29827. In connection, *Dinbergs* teaches that the naturally occurring

release rate for a given growth factor should be considered when choosing the rate to deliver growth factors to cells. *See Dinbergs* at 29826. For TGF-β in particular, *Dinbergs* showed that sustained release from a microsphere was better at inhibiting proliferation of smooth muscle cells. *Dinbergs* at 29822-3. *Dinbergs* is about release and delivery of growth factors. *Dinbergs* is not about tissue engineering scaffolds, and fails to disclose a method of making a tissue engineering scaffold.

1. Dinbergs does not concern the extracellular matrix

Dinbergs fails to disclose "production of extracellular matrix without increasing cellular proliferation," as is recited in Applicants' independent claims 1 and 24. On page 9 of the Final Office Action, the Examiner contends that *Dinbergs* discloses "inducing formation of extracellular matrix" (Final Office Action at 9), and that

> Dinbergs *et al* teach . . . . The reference TGFβ is effective to elicit production of extracellular matrix (see page 29822, column 2, last paragraph, in particular) without increasing cellular proliferation (See Fig 2B, Fig 3B, Abstract, in particular). . . . Dinbergs *et al* teach TGFβ is useful for eliciting extracellular matrix formation without increasing cellular proliferation for up to five days when coupling to various polymer such as alginate hydrogel for a sustained release (See page 29825, Fig. 3A, in particular).

(Final Office Action at 9.) This is simply not the case.[5]

Dinbergs never studied extracellular matrix production. Rather, *Dinbergs* studied how growth factors interact with the extracellular matrix, and how growth factors released from the extracellular matrix affect cell proliferation. In fact, for *Dinbergs's* studies, the cells were removed from their extracellular matrix before adding any growth factors. *See Dinbergs* at 29823 (explaining in the right hand column under the subheading "Extracellular Matrix Incorporation and Release of Growth Factos" that ". . . to remove the solubilized cells, leaving extracellular matrix coating the bottom of the wells."). *Dinbergs* simply does not study using growth factors to increase extracellular matrix production while minimizing cell proliferation.

Furthermore, *Dinbergs's* Figures 3A and 3B make no mention of eliciting extracellular matrix formation at all. What is mentioned is that TGF-β can inhibit the proliferation of certain

[5] If it were the case, one would be left to wonder why the Examiner has not applied *Dinbergs* as an anticipating reference. However, for the reasons set forth above, *Dinbergs* clearly does not anticipate the claimed invention.

cells. But with reference to smooth muscle cells, *Dinbergs* actually demonstrates cell ***proliferation***. This is clearly seen from Figure 3B, in which the cell number increased after a single day. And by the second day of the experiment, about an additional 25,000 cells have proliferated. Accordingly, *Dinbergs* does not teach that administration of TGF-β may enhance extracellular matrix production without increasing proliferation.

2. Dinbergs does not teach coupling

Dinbergs fails to disclose "covalently coupling matrix-enhancing molecules to the scaffold," as is recited in claim 1. The Examiner contends that

> Dinbergs *et al* teach . . . coupling various matrix-enhancing molecule such as bFGF or TGFβ in a concentration 1-10 ng/ml (See Alginate/Heparin-Sepharose Microsphere Preparation and Growth Factor Incorporation, page 29823, column 2, bridging page 29824 column 1, in particular). . . . Dinbergs *et* al teach TGFβ has been incorporated into scaffold or various biodegradable polymer matrix such as collagen, hydrogel such as alginate, hydron (hyaluronic acid) and polyethylene glycol polymers (See page 29827, column 2, first full paragraph, in particular).

(Final Office Action at 9.) Again, *Dinbergs* does not teach this.

The growth factors in *Dinbergs* are not coupled to a polymeric scaffold, but instead are encapsulated by a polymeric matrix and released as soluble growth factors. Furthermore, the TGF-β used in Figures 3A and 3B of *Dinbergs* was "solvent-cast within EVAc microspheres," rather than tethered to a scaffold. *See Dinbergs* at 29824, col. 1, 5th full paragraph. The growth factors in *Dinbergs* are not coupled to a polymeric scaffold, but instead are encapsulated by a polymer and released as soluble growth factors, quite unlike the invention of independent claim 1.

3. Dinbergs does not disclose the claimed concentrations

Dinbergs fails to disclose "in a density between 1 and 100 ng TGF-β/ml or in a concentration of between about 4×10^{-6} and 4×10^{-3} nmol/ml," as is recited in claim 1. The Examiner contends that

> Dinbergs *et al* teach . . . coupling various matrix-enhancing molecule such as bFGF or TGFβ in a concentration 1-10 ng/ml (See Alginate/Heparin-Sepharose Microsphere Preparation and Growth Factor Incorporation, page 29823, column 2, bridging page 29824 column 1, in particular).

(Final Office Action at 9.) This exaggerates the concentration found in *Dinbergs*.

At the outset, the 1-10 ng/ml figure cited by the Examiner has nothing to do with *Dinbergs's* microsphere studies. This is the growth factor concentration added to cell-free extracellular matrix for the ECM incorporation and release experiment shown in Figure 5. *See Dinbergs* at 29823, col. 2, fourth full paragraph-29824 ("Extracellular Matrix Incorporation and Release of Growth Factors"); *Id.* at 29825, col. 2, third fill paragraph-29826 ("bFGF and TGF-β1 Release from the Extracellular Matrix").

Dinbergs specifically discloses forming EVAc-BSA-TGF-β1 microspheres by adding TGF-β1 at a concentration of 3 ng TGF-β1 per microsphere. *See Dinbergs* at 29823, col. 2, third full paragraph. And each microsphere releases 0.4 ng TGF-β. *See* 29825, col. 1, second full paragraph ("one EVAc-BSA-TGF-β1 microsphere releas[es] 0.4 ng TGF-β1"); *see also Dinbergs* at 29823, col. 1, fifth full paragraph-29823, col. 2 ("Cell Proliferation Assay"). Even if we incorrectly assume a single microsphere could be a tissue engineering scaffold, the microsphere falls short of the claimed concentration.[6]

4. A microsphere is not a "scaffold"

On page 9 of the Office Action, the Examiner contends:

> Dinbergs *et al* teach a method for making a tissue engineering scaffold such as alginate/heparin-sepharose microsphere . . . .

(Final Office Action at 9.). A microsphere, however, is not a tissue engineering scaffold.

A single microsphere, as disclosed by *Dinbergs*, is not a scaffold.[7] The scaffolds of the present invention are "useful in not only tissue engineering but also for tissue regeneration and wound healing applications." (*See* Application at 3, ll. 3-5.) In contrast, a single microsphere, being a discrete object having a microscopic size, could not by itself be thought capable of having any use in such applications.[8] A scaffold of the present invention may be surgically

[6] The use of more than one of the *Dinbergs's* microspheres could meet the concentration limitation—for example, three of *Dinbergs's* microspheres in one milliliter would have a concentration of 1.2 ng of TGF-β/ml. *Dinbergs*, however, does not teach or suggest using more than one microsphere.

[7] In *Dinbergs*, the microsphere used to encapsulate TGF-β was actually the EVAc microsphere. *Compare Dinbergs* at 29823, col. 2, first full paragraph, *with Dinbergs* at 29823, col. 2, third full paragraph.

[8] Applicants recognize, however, that a tissue engineering scaffold according to Applicants' invention may formed by using a sufficient number of discrete scaffolds to form a larger tissue engineering scaffold so long as the

implanted in the body of a mammal. (*See, e.g.*, Application at 8, ll. 12-13 ("The scaffold is typically seeded with the cells; the cells are cultured, and then the scaffold implanted.").) A single microsphere is not an implantable article; rather, it is used in vitro as taught by *Dinbergs*. A single microsphere would not, ***by itself***, be surgically implanted in the body of a mammal, quite unlike the scaffolds of the present invention, which are designed to be implanted into the body of a mammal. Accordingly, *Dinbergs* should not be considered analogous art.

5. Examiner has taken *Dinbergs* out of context

Furthermore, the Examiner has taken *Dinbergs* out of context. The Examiner asserted that

> Dinbergs *et al.* teach TGFβ has been incorporated into scaffold or various biodegradable polymer matrix such as collagen, hydrogel such as alginate, hydron (hyaluronic acid) and polyethylene glycol polymers (See page 29827, column 2, first full paragraph, in particular).

(Final Office Action at 9-10.) The Examiner has extracted an incomplete quotation that misrepresents the actual teaching of the reference. When the cited portion of *Dinbergs* is considered in context, the Examiner's error becomes obvious. The cited portion of *Dinbergs* actually states:

> Various polymeric devices have been used for the controlled release of a number of growth factor. A commonly used system for growth factor release is ethylene-vinyl acetate copolymer (40, 43, 61, 62, 64, 65, 67, 84-87), although the organic solvents used in the preparation of this device destroy the biologic activity of molecules such as bFGF (18, 22). TGF-β1 has been delivered for the purpose of bone repair via the biodegradable polymer poly-(DL-lactide-co-glycolide) and demineralized bone matrix (69, 71, 70, 73), although problems of immunocompatibility, osteoinductivity, and osteoconductivity exist. Other polymer materials have included poly(methyl methacrylate) (58), Pluronic F-127 poloxamer gel (74), polyethylene glycol (88), collagen (63, 77, 89), methylcellulose (75), chitosan (19, 82), cyclodextrin (20), lipds (90), Hydron (78, 83, 91) and other hydrogels, ***but these all have various limitations such as shorter or suboptimal release times and difficulty of handling***. Microspheres consisting of the biocompatible and biodegradable polymer alginate have also been

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concentration limitation of Applicants' claims are met. Applicants do not disclaim such tissue engineering structures.

> utilized for controlled release delivery of some growth factors. . . . TGF-β1 release from sodium alginate microspheres has been demonstrated as a potential ***oral gastrointestinal drug delivery system, in which TGF-β1 is completely and rapidly released within 2 h after a low pH environment is changed to pH 7.4 (80).*** Our data now support the notion that in considering the burgeoning technology of controlled release, the different interactions these growth factors have with the extracellular matrix must be taken into account. ***Sustained release should be reserved for those growth factors that are naturally sustain-released.***

Dinbergs at 29827, col. 2, first full paragraph (emphasis added). In contrast to the Examiner's application of this portion of *Dinbergs*, the text, in context, actually shows that:

- the prior art did disclose the use of collagen, polyethylene glycol polymers, hydron, and other hydrogels to deliver growth factors, but each one of these materials was found to be problematic, *e.g.*, demonstrating "shorter or suboptimal release times and difficulty in handling." *See id.*

- ***none*** of these disclosures cited in *Dinbergs*, nor even *Dinbergs* itself, disclosed tethering a growth factor to a scaffold, much less covalent tethering. Nowhere in the above excerpt is "covalent", "tethering", or a "scaffold" mentioned. *See id.*

- the embodiment cited by the Examiner involving the use of alginate to release TGF-β actually discloses the "complete" and "rapid" release of TGF-β during a 2 hour period.

- the excerpt explicitly teaches away from the sustained release of growth factors, which constitutes a teaching away from Appellants' invention.

Accordingly, the excerpt from *Dinbergs*, when combined with the '430 Patent, fails to obviate claim 1.

C. Even if the '430 Patent and *Dinbergs* are combined, the combination fails to obviate claim 1

The combination of the '430 Patent and *Dinbergs* is not only improper, but moreover, it fails to obviate claim 1. Neither reference discloses the benefit of enhancing extracellular matrix formation without increasing cellular proliferation, and neither discloses coupling TGF-β to a polymeric scaffold in an effective density between 1-100 ng/mL.

The '430 Patent does not disclose a method of enhancing production of extracellular matrix molecules. Nor does the '430 Patent disclose TGF-β in effective density of 1-100 ng

TGF-β/ml. And the Examiner failed to demonstrate that the '430 Patent-*Dinbergs* combination teaches or suggests a method of enhancing extracellular matrix formation without increasing cellular proliferation, and failed to demonstrate that the combination teaches or suggests coupling TGF-β to a polymeric scaffold in an effective density between 1-100 ng/mL.

Accordingly, even if the cited references are combined, the Examiner has failed to show how the combination obviates claim 1. As claim 1 is nonobvious over the prior art, dependent claims 2 and 8 are similarly nonobvious because they include the limitations of their respective base claim, which Applicants have shown above to be allowable.

VI. The combination of the '430 Patent, *Dinbergs*, and the '849 Patent fails to obviate claims 1, 7, and 8

Not only is the combination of the '430 Patent, *Dinbergs*, and the '849 Patent improper, it also fails to obviate claims 7 and 8. (*See* Final Office Action at 12.) As demonstrated above, the '430 Patent and *Dinbergs* do not obviate Applicants' independent claim 1. The '849 Patent does not teach the subject matter missing from the '430 Patent and *Dinbergs*. As claim 1 is nonobvious over the prior art for the reasons described above, dependent claims 7 and 8 are similarly nonobvious because they include the limitations of their respective base claim, which Applicants have shown above to be allowable.

Moreover, the proposed combination of the '430 Patent, *Dinbergs*, and the '849 Patent is improper because the Examiner has not provided a sufficient teaching, suggestion, or motivation in the prior art to make such a combination, as discussed herein. Such improper combination cannot properly be used to obviate the subject claims.

A. The '849 Patent does not teach the subject matter missing from the '430 Patent and *Dinbergs*

The Examiner has not asserted that the further combination of the '849 Patent obviates claim 1. (*See* Final Office Action at 12.) Nevertheless, Applicants explain below that the '849 Patent does not provide the subject matter missing from the '430 Patent and *Dinbergs*.

1. The '849 Patent fails to disclose several limitations of claim 1

The '849 Patent concerns bioartificial organs (BAOs). BAOs are devices that encapsulate cells within a semi-permeable membrane. '849 Patent, col. 5, ll. 9-40. According to the '849

Patent, the cells encapsulated by the BAO may grow on the inner luminal surface of the membrane, or they may grow on an inner surface encapsulated within the BAO. '849 Patent, col. 16, ll. 43-46. But the '849 Patent does not disclose tethers, matrix-enhancing molecules covalently coupled, or suitable concentrations of TGF-β.

a. The '849 Patent fails to disclose tethers

The '849 Patent fails to disclose "TGF-β is covalently coupled to the matrix by a polymer tether," as recited by claim 1. The Examiner contends, that the '849 Patent teaches "covalently coupling to an inner matrix by a tether such as poly-d-lysine (see col. 18, line 30-35, in particular)." (Final Office Action at 12.) The Examiner is wrong for two reasons. Poly(d-lysine) is not a tether; rather, poly(d-lysine) promotes cellular attachment. '849 Patent col. 18, ll. 30-33. And the '849 Patent does not describe using poly(d-lysine) with a scaffold. *See* '849 Patent, col. 19, ll. 18-24. Instead, the '849 Patent describes adsorbing poly(d-lysine) onto the BAO's membrane. '849 Patent col. 18, ll. 33-35. In contrast, the tethers of the present invention connect the matrix-enhancing molecules to the scaffold. (*See* Application at 7, ll. 1-10.)

b. The '849 Patent fails to disclose covalently coupled TGF-β

The '849 Patent fails to disclose "TGF-β covalently coupled," as recited by claim 1. The Examiner contends that the '849 Patent teaches

> covalently coupling to an inner matrix by a tether such as poly-d-lysine (see col. 18, line 30-35, in particular) coupling to matrix enhancing molecules such as RGD containing sequence (see col. 18, lines 36-51, in particular) or TGF beta and/or ascorbic acid (see col. 12, lines 56-67, in particular).

(Final Office Action at 12-13.) The Examiner has mischaracterized the '849 Patent's teachings.

The '849 Patent does not teach or suggest covalently coupling TGF-β. Instead, the '849 Patent teaches that "cells may be exposed to a treatment which inhibits proliferation or induces differentiation" using a "chemical compound or growth factor." '849 Patent col. 11, ll. 56-63 & col. 24, ll. 50-58 ("BHK cells secreting hNGF were treated with 2.5 ng/ml TGFβ and 100 μm ascorbic acid prior to encapsulation in BAOs and implantation."). The portion of the '849 Patent concerning TGF-β cited by the Examiner only refers to "exposure," and makes no mention whatsoever of coupling, much less "covalently coupling." '849 Patent col. 12, ll. 59-62 ("For

example, decreased proliferation and enhanced differentiation in BHK cells can be achieved by ***exposure*** to TGFβ1 and ascorbate." (emphasis added)).

c. The '849 Patent fails to disclose the claimed concentration of TGF-β

The '849 Patent fails to disclose TGF-β "in a density between 1 and 100 ng TGF-β/mL or in a concentration of between about 4×10^{-6} and 4×10^{-3} nmol/ml," as recited by claim 1. In fact, the '849 Patent makes no mention of stimulating ECM production by cells associated with a tissue engineering scaffold. *See* '849 Patent col. 14, l. 23-col. 15, l. 67 (describing the use of ECM molecules to control cell growth and differentiation). And although the '849 Patent does describe a number of matrix-enhancing molecules, it does not disclose any concentration suitable for the claimed invention. *See* '849 Patent, col. 11, l. 54- col. 14, l. 6. In fact, portions of the '849 Patent are specifically directed to promoting cellular proliferation, which teaches away from the claimed invention. *See* '849 Patent col. 20, ll. 42-50 ("The core can comprise a liquid medium sufficient to maintain cell growth.").

B. There is no suggestion in either the '430 Patent, *Dinbergs* or '849 Patent to incorporate the teachings of the other

1. The '430 Patent, *Dinbergs*, and '849 Patent are incompatible and cannot be combined

The '849 Patent teaches using unbound chemical compounds or growth factors that inhibit cell proliferation or induce differentiation to control cell distribution within a BAO. As shown above, the '430 Patent teaches away from the claimed invention because it concerns cell proliferation, ***the very thing that the claimed invention avoids***; and *Dinbergs* teaches away from the claimed invention because it concerns release of soluble growth factors, which is in direct conflict with claim 1's requirement to covalently couple TGF-β to the scaffold. Because they teach different outcomes, the '430 Patent, *Dinbergs*, and '849 Patent are incompatible and cannot be combined. The inclusion of the '849 Patent in the obviousness rejections represents clear error that must be reversed.

The teachings of the '430 Patent and *Dinbergs* are described above. (*See* supra Section V.) The '849 Patent teaches that proliferation-inhibiting or differentiation-inducing compounds, like TGF-β, may used to control cell distribution within a BAO. '849 Patent col. 12, ll. 46-51. In connection, the '849 Patent teaches exposing cells to proliferation-inhibiting compounds, like

TGF-β. '849 Patent col. 12, ll. 57-62. The Examiner contends that "ascorbic acid and TGFbeta1 increase collagen biosynthesis as taught by the '849 patent (see col. 12, line 57-67, Table 1, in particular)." (Final Office Action at 13.) Table 1 however, describes how ECM molecules, growth factors, and chemical compounds influence proliferation or differentiation of various cell types. There is nothing in Table 1, or the text of the '849 Patent, that teaches increased collagen biosynthesis by ascorbic acid and TGF-β. For ascorbic acid and TGF-β, what is disclosed is that together these promote the proliferation of neuroendocrine cells and inhibit BHK cells. *See* '849 Patent col. 16, Table 1. The '849 Patent makes no reference to increased collagen biosynthesis.

In light of these teachings, a combination of the '430 Patent, *Dinbergs*, and the '849 Patent would change the basic principle under which each was designed to operate. The '430 Patent was designed for sustained release of growth factors, such as TGF-β, to "stimulate tissue growth to a detectable degree." '430 Patent, col. 7, ll. 18-22; col. 19, ll. 10-56 and Figure 2. But according to the '849 Patent and *Dinbergs*, exposure to TGF-β would actually inhibit cellular proliferation, preventing tissue growth to a detectable degree. Therefore, there would be no motivation to combine the teachings of the '430 Patent, *Dinbergs*, and the '849 Patent.

2. The proposed '430 Patent-*Dinbergs*-'849 Patent combination has no reasonable expectation of successfully achieving the invention

Not only does the above-described incompatibility of the '430 Patent and *Dinbergs* with the '849 Patent preclude a suggestion or motivation to combine, the combined teachings of these references fail to provide an ordinary artisan with any, much less a reasonable, expectation of successfully making the invention.

As discussed above, the '849 Patent teaches that the use of proliferation-inhibiting compounds in the '430 Patent would not succeed in stimulating tissue growth, because exposure to these compounds would actually inhibit cellular proliferation, preventing tissue growth to a detectable degree. And the '430 Patent teaches that cellular proliferation is what is needed to stimulate tissue growth to a detectable degree. Similarly, the '849 Patent teaches that cells may be exposed to TGF-β before being encapsulated in a BAO. This is not compatible with the teachings of *Dinbergs*, which teaches release of soluble growth factors like TGF-β. Because the teachings of the '430 Patent, *Dinbergs*, and the '849 Patent are incompatible, a skilled artisan would have no reason to expect that the proposed '430 Patent-*Dinbergs*-'849 Patent combination

would be successful to elicit production of extracellular matrix without increasing cellular proliferation.

C. Even if the '430 Patent, *Dinbergs*, and '849 Patent are combined, the combination fails to obviate claim 1

Even if the '430 Patent, *Dinbergs*, and the '849 Patent could properly be combined, such a combination would fail to obviate claim 1. None of the references alone or in combination discloses enhancing extracellular matrix formation without increasing cellular proliferation, or the benefit of doing so. Indeed, at an even more basic level neither reference teaches or suggests "covalently coupling the matrix-enhancing molecules to the scaffold in an effective density to elicit production of extracellular matrix without increasing cellular proliferation," as recited in independent claim 1. As discussed above in Section V.C, the '430 Patent and *Dinbergs* have not been shown to teach this limitation. Nor has the '849 Patent been shown to supply this missing limitation. Nowhere has the '849 Patent been shown to discuss suitable concentrations of matrix-enhancing molecules, much less "an effective density to elicit production of extracellular matrix without increasing cellular proliferation." Accordingly, even if the cited references are combined, the Examiner has failed to show how the combination obviates claim 1. As claim 1 is nonobvious over the prior art, dependent claims 7 and 8 are similarly nonobvious.

VII. The combination of the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 fails to obviate the claims 1 and 8

Not only is the combination of the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 improper, it also fails to obviate claim 1. (*See* Final Office Action at 14.) As demonstrated above in Section V, the '430 Patent and *Dinbergs* do not obviate Applicants' independent claim 1. WO 94/23740 and WO 96/27657 do not teach the subject matter missing from the '430 Patent and *Dinbergs*. As claim 1 is nonobvious over the prior art for the reasons described above, dependent claim 8 is similarly nonobvious because it includes the limitations of its respective base claim, which Applicants have shown above to be allowable.

Moreover, the proposed combination of the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 is improper because the Examiner has not provided a sufficient teaching, suggestion, or motivation in the prior art to make such a combination, as discussed herein. Such improper combination cannot properly be used to obviate the subject claims.

A. WO 94/23740 or WO 96/27657 do not teach the subject matter missing from the '430 Patent and *Dinbergs*

The Examiner has not asserted that the further combination of WO 94/23740 or WO 96/27657 obviates claim 1. (*See* Final Office Action at 14.) Nevertheless, Applicants explain below that WO 94/23740 or WO 96/27657 do not provide the subject matter missing from the '430 Patent and *Dinbergs*.

1. WO 94/23740 and WO 96/27657 fail to disclose several limitations of claim 1

a. WO 94/23740 fails to disclose a method of making a tissue engineering scaffold or that matrix enhancing molecules may be coupled to the tissue engineering scaffold.

WO 94/23740 fails to disclose a method of making a tissue engineering scaffold, or that matrix enhancing molecules may be coupled to such a tissue engineering scaffold. The Examiner contends that WO 94/23740 teaches methods of making a tissue engineering scaffold.

> The WO 94/23740 publication teaches a method for making a tissue engineering scaffold comprising coupling various matrix-enhancing molecules such as TGFβ or TGFβ2 covalently coupling to polyethylene glycol (See page 12, line 11, PEG-TGF-β conjugates, rhTGF- TGF-β2 (PEG5000) bridging page 13, in particular). The WO 94/23740 publication teaches the method of making a tissue engineering scaffold comprising coupling TGFβ to a polymer is useful for simulation of bone formation at a lower does (See abstract, in particular).

(Final Office Action at 14.) The Examiner has misinterpreted the WO 94/23740 disclosure. First, "bone formation" is not a "scaffold," it is simply the process by which bone is formed. Indeed, nowhere in the WO 94/23740 publication is the word "scaffold" even mentioned. Rather, WO 94/23740 discloses a method for stimulating bone formation in an animal by administering to the animal an effective amount of a hydrophilic polymer-conjugated growth factor ***in solution***.

The WO 94/23740 simply teaches modifying a growth factor, like TGF-β, by covalently binding a water soluble hydrophilic polymer, like PEG. WO 94/23740 at 5, ll. 8-11, 5, l. 35-6, l. 2. As taught in WO 94/23740, the polymer (e.g., PEG) is not a scaffold. Rather, the polymer is a substance that is conjugated with the growth factor, whereupon the polymer-conjugated growth factor is administered to an animal ***in solution***. *See, e.g.*, WO 94/23740 at 12, ll. 28-33. As taught in WO 94/23740, the polymers disclosed in WO 94/23740 are not themselves scaffolds,

nor does WO 94/23740 even disclose tethering its polymer-conjugated growth factors to a scaffold. As taught in WO 94/23740, the polymers disclosed in WO 94/23740 are ***systemically administered***, whereas the scaffolds of the present invention are ***locally administered***. WO 94/23740 at 16, ll. 24-32. Accordingly, WO 94/23740 does not disclose methods of making a tissue engineering scaffold, much less methods of making a tissue engineering scaffold involving covalently coupling a matrix enhancing molecule like TGF-β to the scaffold, a limitation that is recited in Applicants' claim 1.

b. WO 94/23740 fails to disclose tethering a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation.

WO 94/23740 fails to disclose tethering a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation. WO 94/23740 concerns cell proliferation, in contrast to the present invention. *See* WO 94/23740 20, ll. 7-22 (noting significant increases in proliferation of osteoblast-like cells, which was interpreted as bone stimulation). Nowhere does WO 94/23740 refer to extracellular matrix production. Accordingly, the Examiner has failed to show that WO 94/23740 discloses tethering a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation, as recited in Applicants' claim 1.

c. WO 94/23740 fails to disclose the covalently coupling of TGF-β to a scaffold in an effective density of between 1-100 ng TGF-β/mL

WO 94/23740 fails to disclose covalently coupling of TGF-β to a scaffold in an effective density of between 1-100 ng TGF-β/mL. Examiner has failed to show that the WO 94/23740 publication discloses that, when TGF-β is used as a matrix enhancing molecule, the TGF-β is present in an effective density of between 1-100 ng TGF-β/mL, as recited in Appellants' claim 1. (*See* Final Office Action at 14-15.)

d. WO 96/27657 fails to disclose covalently coupling a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation

WO 96/27657 does not disclose covalently coupling a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation.

According to WO 96/27657, various growth factors are covalently attached to a scaffold via a polymer tether. *See* WO 96/27657 at 10, l. 20-11, l. 10; 10, ll. 3-5; 6, ll. 13-14. The growth factors of WO 96/27657, however, are used at concentrations that stimulate cellular proliferation. *See* WO 96/27657 abstract & claims 1, 12, 31. Accordingly, the Examiner has failed to show that WO 94/23740 discloses covalently coupling a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation, as recited in Applicants' claim 1.

e. WO 96/27657 does not disclose, in the case of TGF-β, an effective density of 1-100 ng TGF-β/ml

WO 96/27657 does not disclose covalently coupling of TGF-β to a scaffold in an effective density of between 1-100 ng TGF-β/mL. Thus, the Examiner has failed to show that WO 96/27657 discloses that, when TGF-β is used as a matrix enhancing molecule, the TGF-β is present in an effective density of between 1-100 ng TGF-β/mL, as recited in Applicants' claim 1. (*See* Final Office Action at 14-15.)

B. There is no suggestion in either the '430 Patent, *Dinbergs*, WO 94/23740 or WO 96/27657 to incorporate the teachings of the other

1. The '430 Patent and WO 94/23740 or WO 96/27657 are incompatible and cannot be combined

As shown above, the '430 Patent concerns cell proliferation, and emphasizes "healing or regrowth of normal tissue." *See, e.g.*, '430 Patent, col. 6, l. 53-col. 7, l. 5. Similarly, WO 94/23740 and WO 96/27657 emphasize cell growth and proliferation. *See, e.g.*, WO 94/23740 at p. 4, ll. 1-19, p. 20, ll. 8-12 ("Very highly significant proliferation of osteoblast-like cells was observed in the femur slides of mice treated at 3 μg with rTGF-β2 $(PEG\ 5000)_6$ OR rTGF-β2 $(PEG\ 5000)_4$ or rTGF-β2 $(PEG\ 35{,}000)_{1\text{-}3}$, as compared with controls."); WO 96/27657 at p.3, ll. 21-22 ("It is therefore an object of the invention to provide a cell and tissue growth substrate that stimulates long-term target cell growth."). In contrast, the present invention discloses, among other things, increasing production of extracellular matrix while ***minimizing*** cell growth. Accordingly, one of ordinary skill attempting to increase production of extracellular matrix without increasing cell growth would not be motivated to combine the teachings of the '430 Patent and WO 94/23740 or WO 96/27657, because these references emphasizes ***increasing*** cell

growth, in direct contrast to the present invention. In fact, the '430 Patent, WO 94/23740, and WO 96/27657 teach away from the claimed invention.

2. *Dinbergs* and WO 94/23740 or WO 96/27657 are incompatible and cannot be combined

As shown above, *Dinbergs* teaches that TGF-β weakly inhibits cellular proliferation, which contrasts with the intent of WO 94/23740 and WO 96/27657, which is to promote cellular proliferation. Because *Dinbergs* teaches the inhibition of cell growth, while WO 94/23740 and WO 96/27657 teach the promotion of cell growth, the references cannot be combined. One of ordinary skill attempting to increase production of extracellular matrix without increasing cell growth would not be motivated to combine the teachings of the *Dinbergs* and WO 94/23740 or WO 96/27657, because these references emphasizes ***increasing*** cell growth, in direct contrast to the present invention.

Moreover, a combination of *Dinbergs* and WO 94/23740 or WO 96/27657 would change the basic principle under which each was designed to operate. As described above, WO 94/23740 and WO 96/27657 were designed to stimulate cell growth and proliferation.. But according to *Dinbergs*, exposure to TGF-β would actually inhibit cellular proliferation, preventing cell growth. Therefore, there would be no motivation to combine the teachings of *Dinbergs* and and WO 94/23740 or WO 96/27657.

The inclusion of the WO 94/23740 and WO 96/27657 in the obviousness rejections represent clear error that must be reversed.

3. The proposed '430 Patent-*Dinbergs*-WO 94/23740 or -WO 96/27657 combination has no reasonable expectation of successfully achieving the invention

Not only does the above-described incompatibility of the '430 Patent and *Dinbergs* with WO 94/23740 or WO 96/27657 preclude a suggestion or motivation to combine, the combined teachings of these references fail to provide an ordinary artisan with any, much less a reasonable, expectation of successfully making the invention.

As discussed above, *Dinbergs* teaches that the use of proliferation-inhibiting compounds in the '430 Patent and WO 94/23740 or WO 96/27657 would not succeed in stimulating tissue growth, because exposure to these compounds would actually inhibit cellular proliferation. Because the teachings of the '430 Patent, *Dinbergs*, and and WO 94/23740 or WO 96/27657 are

incompatible, a skilled artisan would have no reason to expect that the proposed '430 Patent-*Dinbergs*-WO 94/23740 or -WO 96/27657 combination would be successful to elicit production of extracellular matrix without increasing cellular proliferation.

C. Even if the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 are combined, the combination fails to obviate claim 1

Even if the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 could properly be combined, such a combination would fail to obviate claim 1. None of the references alone or in combination discloses enhancing extracellular matrix formation without increasing cellular proliferation, or the benefit of doing so. Indeed, at an even more basic level neither reference teaches or suggests "covalently coupling the matrix-enhancing molecules to the scaffold in an effective density to elicit production of extracellular matrix without increasing cellular proliferation," as recited in independent claim 1. As discussed above, the '430 Patent and *Dinbergs* has not been shown to teach this limitation. Nor has either WO 94/23740 or WO 96/27657 been shown to supply this missing limitation. Nowhere has WO 94/23740 or WO 96/27657 been shown to discuss suitable concentrations TGF-β matrix-enhancing molecules, much less "an effective density to elicit production of extracellular matrix without increasing cellular proliferation." Accordingly, even if the cited references are combined, the Examiner has failed to show how the combination obviates claim 1. As claim 1 is nonobvious over the prior art, dependent claims 7 and 8 are similarly nonobvious.

D. The combination of the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 fails to obviate the additional limitations found in dependent claim 8

At the outset Applicants note that in rejecting claim 8, the Examiner failed to show how the combination of the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 obviates claim 7, from which claim 8 depends. For this reason alone claim 8 has not been shown to be obvious over the combined references. Nevertheless, Applicants explain below that the combined references fail to teach or suggest the additional limitations present in claims 7 and 8.

Not only is the combination of the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 improper, it also fails to teach or suggest the additional limitations present in claims 7 and 8. (*See* Final Office Action at 14-15.)

With respect to claim 8, the Examiner stated:

> The claimed invention in claim 8 differs from the teachings of the references only in that the method wherein the scaffold is a hyaluronic acid or polyethylene glycol polymer instead of collagen.

(Final Office Action at 14.) With respect to claims 7 and 8, Applicants note that neither of WO 94/23740 and WO 96/27657 teaches or suggests the additional limitations of the scaffold being a hydrogel (claim 7), the hydrogel being formed of a polymer selected from the group consisting of alginate, collagen, hyaluronic acid, and polyethylene glycol polymers (claim 8).

As discussed above, the polymers disclosed in WO 94/23740 are not themselves scaffolds, nor does WO 94/23740 even disclose tethering its polymer-conjugated growth factors to a scaffold. In WO 94/23740, the word "scaffold" and "hydrogel" are not even mentioned. Thus, even if the references are combined, the Examiner has failed to show that the combination obviates the additional limitations found in claims 7 and 8.

Although WO 96/27657 discloses "attachment substrates," the Examiner has not shown how WO 96/27657 obviates claims 7 and 8. First, WO 96/27657 makes no mention of a scaffold formed from a hydrogel. The embodiment that the Examiner cites as disclosing "a scaffold such as hyaluronic acid (see page 7, line 1, in particular)" actually discloses a tether, rather than a scaffold. *See* WO 96/27657 at p 6, l. 27-p. 7, l. 2 ("Examples of water-soluble, biocompatible polymers which can serve as tethers include . . . ."). And the embodiment that the Examiner cites as disclosing "polyethylene oxide, or alginate, (See page 17, line 8, in particular)" does not teach or suggest that these polymers are used in the form of a hydrogel. *See* WO 96/27657 at p 9, ll. 8-24. Indeed, WO 96/27657 notes that the attachment substrates may have various forms, but fails to even suggest a hydrogel. *See* WO 96/27657 at p 9, ll. 25-29.

Thus, even if the references are combined, the Examiner has failed to show that the combination obviates the additional limitations found in claims 7 and 8. Moreover, the proposed combination of the '430 Patent, *Dinbergs*, and WO 94/23740 or WO 96/27657 is improper because the Examiner has not provided a sufficient teaching, suggestion, or motivation in the prior art to make such a combination, as discussed above. Such improper combination cannot properly be used to obviate the subject claims.

VIII. The combination of the '430 Patent and the '849 Patent fails to obviate claims 24-27 and 32-34

Claims 24-27 and 32-34 are patentable over the combined teaching of the '430 Patent and the '849 for several reasons. First, the Examiner has improperly construed the scope of claim 24 as explained in detail above. Second, there is no reason, suggestion, or motivation to combine the references in the manner required to produce the claimed invention. Third, even if the references are combined in the manner indicated by the Examiner, the resulting method would not include every limitation recited in Applicants' independent claim 24.

A. The Examiner has improperly construed the scope of claim 24 by ignoring a limitation expressly recited

As explained above in Section III, the Examiner improperly construed the scope of claim 24. In doing so, the Examiner has improperly rejected claims 24-27 and 32-34 as obvious in view of art that clearly does not teach or suggest the claimed method of coupling a matrix-enhancing molecule to a tissue engineering scaffold in an effective density to increase extracellular matrix production ***without*** increasing cellular proliferation. In addition, the Examiner has not shown that the '430 Patent-'849 Patent combination teaches or suggests each and every limitation recited in Applicants' independent claim 24.

B. The Examiner's rejection of claim 24 in view of the '430 Patent-'849 Patent combination is improper

Not only has the Examiner failed to establish a prima facie case of obviousness because the combination of the '430 Patent and '849 Patent has not been shown to teach or suggest each limitation of Applicants' independent claim 24, the Examiner has also improperly combined the '430 Patent and '849 Patent.

1. The '849 Patent fails to disclose several limitations of claim 24

The '849 Patent is discussed as it relates to Applicants' independent claim 1 above in Section VI. The '849 Patent, as it relates to claim 24, does not disclose tethers, matrix-enhancing molecules coupled to a scaffold, or suitable concentrations of matrix-enhancing molecules.

a. The '849 Patent fails to disclose tethers

The '849 Patent fails to disclose "covalently coupling the polymer tether to the scaffold," as recited by claim 24, as is discussed above in Section VI.A.1.a.

b. The '849 Patent fails to disclose that matrix-enhancing molecules may be coupled to the tissue engineering scaffold

The '849 Patent fails to disclose "covalently coupling the matrix-enhancing molecule to the scaffold," as recited by claim 24. The Examiner contends that the '849 Patent teaches

> covalently coupling to an inner matrix by a tether such as poly-d-lysine (see col. 18, line 30-35, in particular) coupling to matrix enhancing molecules such as RGD containing sequence (see col. 18, lines 36-51, in particular) or TGF beta and/or ascorbic acid (see col. 12, lines 56-67, in particular).

(Final Office Action at 16.) The Examiner has mischaracterized the '849 Patent's teachings.

First, RGD containing sequences are not matrix-enhancing molecules. Rather, these sequences are used to promote cellular attachment (*see* Application at 1, ll. 21-28). '849 Patent col. 18, ll. 36-45. This mistake demonstrates how the Examiner's misconstruction of "matrix-enhancing molecules" has resulted in improper rejections.

Second, the '849 Patent does not teach or suggest covalently coupling the matrix-enhancing molecules, as is discussed above in Section VI.A.1.b.

c. The '849 Patent fails to disclose concentrations

The '849 Patent fails to disclose a "concentration sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell," as recited by claim 24. In fact, the '849 Patent makes no mention of stimulating ECM production by cells associated with a tissue engineering scaffold. *See* '849 Patent col. 14, l. 23-col. 15, l. 67 (describing the use of ECM molecules to control cell growth and differentiation). And although the '849 Patent does describe a number of matrix-enhancing molecules, it does not disclose any concentration suitable for the claimed invention. *See* '849 Patent, col. 11, l. 54- col. 14, l. 6. In fact, portions of the '849 Patent are specifically directed to promoting cellular proliferation, which teaches away from the claimed invention. *See* '849 Patent col. 20, ll. 42-50 ("The core can comprise a liquid medium sufficient to maintain cell growth.").

2. There is no suggestion in either the '430 Patent or '849 Patent to incorporate the teachings of the other

a. The '430 Patent and '849 Patent are incompatible and cannot be combined

As shown above, the '430 Patent teaches away from the claimed invention because it concerns cell proliferation. Because they teach different outcomes, the '430 Patent and '849 Patent are incompatible and cannot be combined.

The '849 Patent teaches using chemical compounds or growth factors that inhibit cell proliferation or induce differentiation to control cell distribution within a BAO. (*See* supra Section VI.B.1.) In contrast, the '430 Patent teaches cellular proliferation. (*See* supra Section V.A.) Because one reference teaches the inhibition of cell growth, while the other reference teaches the promotion of cell growth, the '430 Patent and '849 Patent are incompatible and cannot be combined. (*See* supra Section VI.B.)

In light of these teachings, a combination of the '430 Patent and the '849 Patent would change the basic principle under which each of the '430 Patent was designed to operate. The '430 Patent was designed for sustained release of growth factors, such as TGF-β, to "stimulate tissue growth to a detectable degree." '430 Patent, col. 7, ll. 18-22; col. 19, ll. 10-56 and Figure 2. But according to the '849 Patent, exposure to TGF-β would actually inhibit cellular proliferation, preventing tissue growth to a detectable degree. Therefore, there would be no motivation to combine the teachings of the '430 Patent and the '849 Patent.

b. The proposed '430 Patent-'849 Patent combination has no reasonable expectation of successfully achieving the invention

Not only does the above-described incompatibility of the '430 Patent with the '849 Patent preclude a suggestion or motivation to combine, that the combined teachings of these references fail to provide an ordinary artisan with any, much less a reasonable, expectation of successfully making the invention.

As discussed above, the '849 Patent teaches that the use of proliferation-inhibiting compounds in the '430 Patent would not succeed in stimulating tissue growth, because exposure to these compounds would actually inhibit cellular proliferation, preventing tissue growth to a detectable degree. And the '430 Patent teaches that cellular proliferation is what is needed to stimulate tissue growth to a detectable degree. Because the teachings of the '430 Patent and the

'849 Patent are incompatible, a skilled artisan would have no reason to expect that the proposed '430 Patent-'849 Patent combination would be successful to elicit production of extracellular matrix without increasing cellular proliferation.

3. Even if the '430 Patent and '849 Patent are combined, the combination fails to obviate claim 24.

Even if the '430 Patent and '849 Patent could properly be combined, such a combination would fail to obviate claim 24. Neither reference alone, or in combination, discloses enhancing extracellular matrix formation without increasing cellular proliferation, or the benefit of doing so. Indeed, at an even more basic level neither reference teaches or suggests "covalently coupling the matrix-enhancing molecule to the scaffold, wherein the matrix-enhancing molecule is present at a concentration sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell," as recited in independent claim 24. As discussed above, the '430 Patent has not been shown to teach this limitation. Nor has the '849 Patent been shown to supply this missing limitation. Nowhere has the '849 Patent been shown to discuss suitable concentrations of matrix-enhancing molecules, much less "a concentration sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell." Accordingly, even if the cited references are combined, the Examiner has failed to show how the combination obviates claim 24. As claim 24 is nonobvious over the prior art, dependent claims 25-27 and 32-34 are similarly nonobvious.

IX. The combination of the '430 Patent, the '849 Patent, and *Dinbergs* fails to obviate the additional limitations found in dependent claims 27 and 29

Not only is the combination of the '430 Patent, the '849 Patent, and *Dinbergs* improper, it also fails to teach or suggest the additional limitations present in claims 27 and 29. (*See* Final Office Action at 19.) As demonstrated above, the '430 Patent and '849 Patent do not obviate Applicant's independent claim 24. The further combination of *Dinbergs* does not obviate claim 24, nor dependent claims 27 and 29.

With respect to claim 27, Applicants note that none of the references teach providing a smooth muscles cell attached to the scaffold. And as Applicants have previously shown, *Dinbergs* demonstrates increased proliferation of smooth muscles cells, while making no

mention of eliciting extracellular matrix formation at all. *See Dinbergs* at 29825, Fig. 3B. Thus, even if the references are combined, the Examiner has failed to show that the combination obviates the additional limitations found in claim 27.

With respect to claim 29, Applicants note that none of the references teach the matrix-enhancing molecule being TGF-β coupled to the scaffold at the claimed concentration. As Applicants have previously pointed out, *Dinbergs* actually discloses a concentration for TGF-β far below what Applicants have claimed. Accordingly, even if the references are combined, the Examiner has failed to show that the cited combination obviates the additional limitations found in claim 29.

Moreover, the proposed combination of the '430 Patent, the '849 Patent, and *Dinbergs* is improper because the Examiner has not provided a sufficient teaching, suggestion, or motivation in the prior art to make such a combination, as discussed above in Section VI.B. Such improper combination cannot properly be used to obviate the subject claims.

X. The combination of the '430 Patent and *Scott-Burden* fails to obviate claims 24 and 30

Claims 24 and 30 are patentable over the combined teaching of the '430 Patent and *Scott-Burden*—there is no reason, suggestion, or motivation to combine the references in the manner required to produce the claimed invention; and, even if the references are combined in the manner indicated by the Examiner, the resulting method would not include every limitation recited in Applicants' independent claim 24, much less the additional feature of claim 30.

A. The '430 Patent- *Scott-Burden* combination is improper

Neither the '430 Patent nor *Scott-Burden* suggest that angiotensin II would elicit extracellular matrix production without increasing cellular proliferation. As shown above, the '430 Patent concerns cell proliferation. Thus, growth factors, such as insulin-like growth factor and TGF-β, may be used at concentrations sufficient "to stimulate tissue growth to a detectable degree." '430 Patent, col. col. 6, ll. 58-63, col. 7, ll. 19-20. *Scott-Burden* teaches that angiotensin II is "capable of stimulating both growth and matrix elaboration by cultured vascular smooth muscle cells." *Scott-Burden* at S36. Maintenance of vascular smooth muscle cells in their proliferative form for a prolonged period leads to vascular thickening and vessel occlusion. *Scott-Burden* at S36. Therefore, *Scott-Burden* sought to understand how the vasoconstrictor

angiotensin II impacts both cellular proliferation and the modulation of extracellular matrix. *Scott-Burden* at S37. Their findings demonstrate that angiotensin II stimulates *both* cellular proliferation and production of extracellular matrix. *Scott-Burden* at S36, S40; (*see also*, Final Office Action at 11 ("Scott-Burden et al teach angiotensin II activates the synthesis of extracellular matrix such as glycopeptides and proteoglycans by smooth muscle cells and ***growth*** of smooth muscle cell (see abstract, in particular)." (emphasis added)).

In light of these teachings, the '430 Patent-*Scott-Burden* combination would change the basic principle under which each reference was designed to operate. Namely, the teachings of *Scott-Burden* suggest that the substitution of angiotensin II for TGF-β in the invention of the '430 Patent would result in increased cellular proliferation along with increased production of extracellular matrix. Neither reference discloses or suggests that it is desirable or possible to use angiotensin II, in substitution for TGF-β , to result in production of extracellular matrix without a concomitant increase in cellular proliferation (as required by Applicants' independent claim 24 and dependent claim 30). Therefore, these prior art references fail to provide a reason why one of ordinary skill in the art would have been motivated to combine the teachings of the '430 Patent and *Scott-Burden*, much less how such a combination would arrive at the claimed invention.

Furthermore, the nature of the problem solved by the claimed invention is to stimulate production of extracellular matrix without additionally stimulating cellular proliferation. When each cited reference is considered as a whole, both disclose use of biologically active factors to stimulate tissue growth. Therefore, it would not have been desirable, and thus not obvious, to one of ordinary skill in the art at the time of the invention was made to combine the teachings of the '430 Patent and *Scott-Burden* in order to address how to stimulate extracellular matrix production *without* a concomitant increase in cellular proliferation. Therefore, the combination of the '430 Patent and *Scott-Burden* is improper.

B. Even if the '430 Patent and *Scott-Burden* are combined, the combination fails to obviate claim 24

As Applicants have shown above in Sections IV and VIII, the '430 Patent fails to teach or suggest a matrix-enhancing molecule "present at a concentration sufficient to elicit production of extracellular matrix by the cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell," as recited in Applicants' independent claim 24. The *Scott-Burden* reference does not provide this missing limitation. *Scott-Burden* provides that "Ang

II was capable of ***stimulating both growth and matrix elaboration*** by cultured vascular smooth muscle cells." *See Scott-Burden* at S36, Abstract (emphasis added). Thus, the proposed combination fails to teach a concentration of matrix enhancing molecule sufficient to elicit extracellular matrix production ***without*** increasing cellular proliferation. Therefore, the proposed combination of the '430 Patent and *Scott-Burden* does not render the claimed invention obvious. As claim 24 is nonobvious over the prior art, dependent claim 30 is similarly nonobvious because it includes the limitations of its respective base claim, which Applicants have shown above to be allowable.

XI. The combination of the '430 Patent and WO 94/23740 or WO 96/27657 fails to obviate claims 24 and 34

Claims 24 and 34 are patentable over the combined teaching of the '430 Patent and WO 94/23740 or WO 96/27657—there is no reason, suggestion, or motivation to combine the references in the manner required to produce the claimed invention; and, even if the references are combined in the manner indicated by the Examiner, the resulting method would not include every limitation recited in Applicants' independent claim 24.

A. The '430 Patent-WO 94/23740 or -WO 96/27657 combination is improper

As shown above in Sections VII.B.1, the '430 Patent and WO 94/23740 and WO 96/27657 both concern cell proliferation. In contrast, the present invention discloses, among other things, increasing production of extracellular matrix while minimizing cell growth. Accordingly, one of ordinary skill attempting to increase production of extracellular matrix without increasing cell growth would not be motivated to combine the teachings of the '430 Patent and WO 94/23740 or WO 96/27657, because these references emphasizes increasing cell growth, in direct contrast to the present invention.

1. WO 94/23740 and WO 96/27657 fail to disclose several limitations of claim 24

a. WO 94/23740 fails to disclose a method of making a tissue engineering scaffold or that matrix enhancing molecules may be coupled to the tissue engineering scaffold.

WO 94/23740 fails to disclose a method of making a tissue engineering scaffold, or that matrix enhancing molecules may be coupled to such a tissue engineering scaffold. As with claim

1, the Examiner contends that WO 94/23740 teaches methods of making a tissue engineering scaffold.

> The WO 94/23740 publication teaches a method for making a tissue engineering scaffold comprising coupling various matrix-enhancing molecules such as TGFβ or TGFβ2 covalently coupling to polyethylene glycol (See page 13, line 11, PEG-TGF-β conjugates, rhTGF- TGF-β2 (PEG5000) bridging page 13, in particular). The WO 94/23740 publication teaches the method of making a tissue engineering scaffold comprising coupling TGFβ to a polymer is useful for simulation of bone formation at a lower does (See abstract, in particular).

(Final Office Action at 24.) The Examiner has misinterpreted the WO 94/23740 disclosure, and WO 94/23740 does not disclose methods of making a tissue engineering scaffold—much less methods of making a tissue engineering scaffold involving coupling a matrix enhancing molecule to the scaffold, a limitation that is recited in Appellants' claim 24. (*See* supra Section VII.A.1.a.)

b. WO 94/23740 fails to disclose tethering a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation.

WO 94/23740 fails to disclose tethering a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation. And the Examiner has failed to show that WO 94/23740 discloses tethering a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation, as recited in Applicants' claim 24. (*See* supra Section VII.A.1.b.)

c. WO 96/27657 fails to disclose covalently coupling a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation

WO 96/27657 does not disclose covalently coupling a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation. The Examiner has failed to show that WO 94/23740 discloses tethering a matrix enhancing molecule to a scaffold to increase extracellular matrix production without increasing cellular proliferation, as recited in Appellants' claim 24. (*See* supra Section VII.A.1.d.)

2. WO 94/23740 and WO 96/27657 both teach away from the present invention

Like the '430 Patent, WO 94/23740 and WO 96/27657 concern cell proliferation, which teaches away from the claimed invention. (*See also* supra Section VII.B.) Indeed, cell growth is expressly stated to be an object of the WO 96/27657 invention. *See* WO 96/27657 at 3, ll. 21-27. And "stimulating bone formation" is described in WO 94/23740. *See, e.g.*, WO 94/23740 at 4, ll. 1-19; *Id.* at 20, ll. 7-22 (noting significant increases in proliferation of osteoblast-like cells, which was interpreted as bone stimulation). Thus, WO 94/23740 and WO 96/27657 teach away from the present invention, which discloses, among other things, increasing production of extracellular matrix while ***minimizing*** cell growth. The inclusion of WO 94/23740 and WO 96/27657 in the obviousness rejections represents clear error that must be reversed.

B. The '430 Patent-WO 94/23740 or -WO 96/27657 combination fails to obviate claim 24

Applicants have demonstrated that the '430 Patent-WO 94/23740 or -WO 96/27657 combination fails to obviate Applicants' claimed invention. Neither reference discloses the coupling of matrix-enhancing molecules to a tissue engineering scaffold, nor discloses coupling matrix-enhancing molecules in an effective density to enhance extracellular matrix production without increasing cellular proliferation. Thus, the proposed combination fails to teach a concentration of matrix enhancing molecule sufficient to elicit extracellular matrix production without increasing cellular proliferation. Therefore, the proposed combination of the '430 Patent and WO 94/23740 or WO 96/27657 does not render the claimed invention obvious. As claim 24 is nonobvious over the prior art, dependent claim 34 is similarly nonobvious.

XII. The claimed methods have produced unexpected results in view of the prior art

Applicants' combination of immobilized growth factor technology and the inhibitory properties of TGF-β results in unexpectedly improved methods that involve enhancing extracellular matrix formation without an increase in cell proliferation. The cited prior art, as discussed above shows or describes increases in cellular proliferation when matrix-enhancing molecules, like TGF-β, are introduced to the cell population. For example: the '430 Patent concerns stimulating tissue growth to a detectable degree (col. 7, ll. 17-22); *Dinbergs* argues that TGF-β1 should not be released in a sustained manner because of its reduced efficacy in

inhibiting cell growth, citing an 18.0-fold increase in endothelial cell number over the original plating density and a 115.0-fold increase for smooth muscle cells (*see* p. 29825, col. 1, last paragraph, bridging col. 2); *Scott-Burden* showed angiotensin II could stimulate proliferation and ECM production (*see* S36, Abstract); WO 94/23740 notes significant increases in proliferation of osteoblast-like cells (*see* p. 20, ll. 7-22); and WO 96/27657 is directed to methods and compositions for stimulating eukaryotic cell growth (*see* abstract & claims 1, 13, 31).

In many tissue engineering applications it is important to avoid undesirable enhancement of cell growth. For example, in vascular tissue engineering, over-proliferation of the smooth muscle cells can lead to a failure of the tissue engineering construct due to lumenal narrowing. Applicants have devised methods that involve increasing extracellular matrix production without increasing cellular proliferation, by coupling matrix-enhancing molecules to a scaffold in an effective density. This is unexpected in light of the prior art: the '430 Patent discloses tethering TGF-β for sustained administration (col. 12, l. 63-col. 13, l. 1); *Dinbergs* teaches TGF-β sustain-released from microspheres is unable to effectively inhibit cell proliferation; the '849 Patent discloses replenishing growth factors, but does not disclose covalent coupling or increasing ECM (col. 13, 64-col. 14, l. 6); *Scott-Burden* discloses angiotensin II's ability to stimulate proliferation and ECM production (*see* S36, Abstract); WO 94/23740 discloses a soluble polymer-conjugated growth factor that increases cellular proliferation; and WO 96/27657 discloses growth factors coupled to scaffolds that increase cellular proliferation.

Applicants' data illustrate an increase in extracellular matrix formation without a corresponding increase in cellular proliferation. (*See* Application Figures 5 & 6.) This result is unexpected in view of the prior art and therefore not obvious to one with ordinary skill in the art.

XIII. Conclusion and requested relief

Appellants have demonstrated that the present invention, as claimed, is enabled and supported by the written description, and is clearly distinguishable over the prior art cited by the Examiner. Therefore, Appellants respectfully request the Board to reverse the final rejections and instruct the Examiner to issue a Notice of Allowance with respect to claims 1-2, 7-8, and 24-35.

Respectfully submitted,
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Date: January 31, 2007

CLAIMS APPENDIX

Listing of Claims:

Claim 1 (Previously Presented): A method for making a tissue engineering scaffold for inducing formation of extracellular matrix by cells bound to the scaffold comprising covalently coupling matrix-enhancing molecules to the scaffold in an effective density to elicit production of extracellular matrix without increasing cellular proliferation, wherein when the matrix-enhancing molecules are TGF-β, the TGF-β is covalently coupled to the matrix by a polymer tether having a molecular weight between 2000 and 6000 and is in a density between 1 and 100 ng TGF-β/ml or in a concentration of between about 4×10^{-6} and 4×10^{-3} nmol/ml.

Claim 2 (Original): The method of claim 1 further comprising attaching cells to the scaffold.

Claim 3 (Original): The method of claim 1 wherein the matrix-enhancing molecules are angiotensin II.

Claim 4 (Original): The method of claim 1 wherein the matrix-enhancing molecules are insulin-like growth factor.

Claim 5 (Original): The method of claim 1 wherein the matrix-enhancing molecules are ascorbic acid.

Claim 6 (Cancelled).

Claim 7 (Original): The method of claim 1 wherein the scaffold is a hydrogel.

Claim 8 (Original): The method of claim 7 wherein the hydrogel is formed of a polymer selected from the group consisting of alginate, collagen, hyaluronic acid, and polyethylene glycol polymers.

Claim 9 (Original): The method of claim 7 wherein the matrix-enhancing molecules are TGF-β coupled to the hydrogel in a concentration of between about 4×10^{-6} and 4×10^{-3} nmol/ml.

Claims 10-23 (Cancelled).

Claim 24 (Previously Presented): A method for making a tissue engineering scaffold, the method comprising:

providing a scaffold, a polymer tether, and a matrix-enhancing molecule;

covalently coupling the polymer tether to the scaffold; and

covalently coupling the matrix-enhancing molecule to the scaffold, wherein the matrix-enhancing molecule is present at a concentration sufficient to elicit production of extracellular matrix by a cell attached to the tissue engineering scaffold without increasing cellular proliferation of the attached cell.

Claim 25 (Previously Presented): The method of claim 24 further comprising providing a cell attached to the tissue engineering scaffold.

Claim 26 (Previously Presented): The method of claim 24 further comprising providing a cell attached to the tissue engineering scaffold, wherein the cell is attached to the tissue engineering scaffold by constraining the cell within the scaffold.

Claim 27 (Previously Presented): The method of claim 24 further comprising providing a cell attached to the tissue engineering scaffold, wherein the cell is selected from the group consisting of smooth muscle cells, endothelial cells, fibroblasts, chondrocytes, and combinations thereof.

Claim 28 (Previously Presented): The method of claim 24 wherein the matrix enhancing molecule is TGF-β.

Claim 29 (Previously Presented): The method of claim 24 wherein the matrix enhancing molecule is TGF-β and the TGF-β is present at a density of between 1 and 100 ng TGF-β/ml or in a concentration of between about 4×10^{-6} and 4×10^{-3} nmol/ml.

Claim 30 (Previously Presented): The method of claim 24 wherein the matrix-enhancing molecule is angiotensin II.

Claim 31 (Previously Presented): The method of claim 24 wherein the matrix-enhancing molecule is insulin-like growth factor.

Claim 32 (Previously Presented): The method of claim 24 wherein the matrix-enhancing molecule is ascorbic acid.

Claim 33 (Previously Presented): The method of claim 24 wherein the scaffold is a hydrogel.

Claim 34 (Previously Presented): The method of claim 24 wherein the scaffold is a hydrogel comprising a polymer selected from the group consisting of alginate, collagen, hyaluronic acid, polyethylene glycol polymers, and combinations thereof.

Claim 35 (Previously Presented): The method of claim 24 wherein the polymer tether has a molecular weight between 200 and 10,000.

EVIDENCE APPENDIX

- Patent No. 5,162,430 ('430 Patent).
- Dinbergs, et al. "Cellular Response to Transforming Growth Factor-β1 and Basic Fibroblast Growth Factor Depends on Release Kinetics and Extracellular Matrix Interactions." J. Biol. Chem. 271:47 29822-29 (1996).
- U.S. Pat. No. 5,935,849 ('849 Patent)
- Scott-Burden, et al. "Modulation of Extracellular Matrix by Angiotensin II: Stimulated Glycoconjugate Synthesis and Growth in Vascular Smooth Muscle Cells." J. Cardiovasc. Pharmacol. 16:suppl. 4 S36-41 (1990).
- WO 94/23740
- WO 96/27657

US005126430A

United States Patent [19]

Senga et al.

[11] Patent Number: 5,126,430

[45] Date of Patent: Jun. 30, 1992

[54] **PROCESS FOR PREPARING POLYARYLENE SULFIDES WITH METAL SALT OF HYDROXYCARBOXYLIC ACID**

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[73] Assignee: **Idemitsu Petrochemical Company Limited**, Tokyo, Japan

[21] Appl. No.: **387,750**

[22] Filed: **Aug. 1, 1989**

[30] **Foreign Application Priority Data**

Aug. 4, 1988 [JP] Japan 63-195575
Sep. 12, 1988 [JP] Japan 63-228273

[51] **Int. Cl.**[5] **C08G 75/16**
[52] **U.S. Cl.** **528/388**; 528/226; 528/387
[58] **Field of Search** 528/388, 387

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,354,129	11/1967	Edmonds, Jr. et al.	260/79
3,867,356	2/1975	Campbell .	
3,869,433	3/1975	Campbell	260/79.1
3,876,591	4/1975	Campbell	260/79.1
3,919,177	11/1975	Campbell	260/79.1
4,096,132	6/1978	Edmonds, Jr. .	
4,116,947	9/1978	Edmonds, Jr. et al.	528/388
4,663,431	5/1987	Fujii et al. .	
4,910,294	3/1990	Ogata et al.	528/388

FOREIGN PATENT DOCUMENTS

59-22926	2/1984	Japan .
61-7332	1/1986	Japan .
63-170422	7/1988	Japan .

OTHER PUBLICATIONS

English Abstract of DE 3,725,997 (Feb. 11, 1988).

Primary Examiner—Harold D. Anderson
Attorney, Agent, or Firm—Nikaido, Marmelstein, Murray & Oram

[57] **ABSTRACT**

A process for preparing a polyarylene sulfide involves contacting a sulfur source with a metallic salt of an aliphatic ω-hydroxycarboxylic acid and a dihalogen aromatic compound in an organic polar solvent, thereby carrying out polycondensation with stability in a polymerization system in which a water content is large. the polyarylene sulfide prepared has a high degree of whiteness and with a sufficiently high molecular weight. The use of the metallic salt of ω-hydroxycarboxylic acid in a particular range can provide a novel polyarylene sulfide.

5 Claims, 1 Drawing Sheet

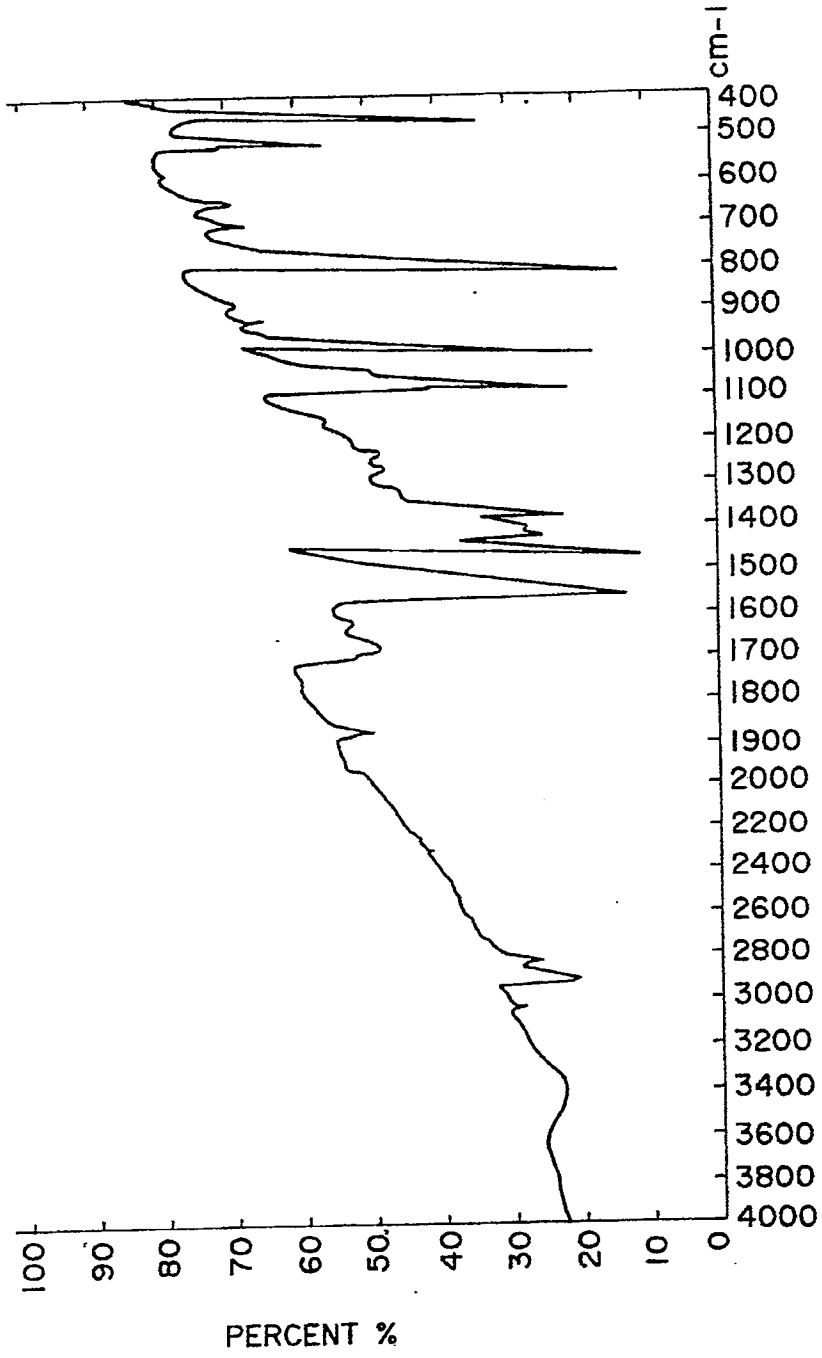
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FIG.1

PROCESS FOR PREPARING POLYARYLENE SULFIDES WITH METAL SALT OF HYDROXYCARBOXYLIC ACID

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a process for preparing a polyarylene sulfide and to a novel polyarylene sulfide prepared thereby.

More particularly, the present invention relates to a process for preparing a polyarylene sulfide, in which a hydrous alkali metal sulfide commercially available is used without dehydration and a polymerization promoter can be recovered with ease. The polyarylene sulfide produced is high in its degree of whiteness, the molecular weight of the polyarylene sulfide so produced is sufficiently high, and it is suitable for molding materials for various molded articles, films, fibers, and mechanical, electric and electronic parts.

The present invention can prevent color from being imparted, by the presence of by-products and the like, to the polyarylene sulfide containing a carbonyl group while permitting the high recovery rate of an organic polar solvent, the polyarylene sulfide of this invention being different in solubility from usual polyarylene sulfides. The process can allow the efficient production of the polyarylene sulfide with a high degree of whiteness in high yields.

Furthermore, the novel polyarylene sulfide according to the present invention can be prepared under specified production condition, it is high in its degree of whiteness, it has a resistance to heat as high as the usual polyarylene sulfides, and it is different in solubility from the usual polyarylene sulfides.

2.Description of Related Art

The polyarylene sulfides such as polyphenylene sulfide are thermoplastic while being partially thermosetting, and they have superior properties as engineering plastics, such as excellent resistance to chemicals, favorable mechanical strength over a broad temperature range, and good thermal rigidity.

The polyarylene sulfides such as polyphenylene sulfide have been heretofore prepared by various processes.

U.S. Pat. No. 3,354,129 proposes a process for preparing polyarylene sulfides in which a polyhalogen aromatic compound, such as a dihalogen aromatic compound, is reacted with an alkali metal sulfide in an organic polar solvent.

U.S. Pat. No. 3,919,177 and Japanese Patent Publication (Kokoku) No. 12,240/1977 propose processes for preparing polyarylene sulfides having a high intrinsic viscosity and low melt flowability, in which a p-dihalobenzene is polymerized with an alkali metal sulfide or an alkali metal bisulfide (an alkali metal hydrosulfide, or an alkali metal hydrogen sulfide) as a sulfur source or another appropriate sulfur compound (for example, thiourea, thioamide, thiocarbamate, thiocarbonate, mercaptan, mercaptide, mercaptocarboxylic acid, or the like) to be used with at least one member of a base, in the presence of a promoter such as a carboxylate (a carboxylic acid of the carbohydryl system having from 1 to 20 carbon atoms), e.g., sodium acetate or lithium acetate, in an organic amide.

U.S. Pat. No. 4,116,947 discloses a process for preparing branched polyarylene sulfides having a relatively high molecular weight by contacting a p-dihalobenzene and a polyhalogen aromatic compound with an alkali metal sulfide in the absence or presence of a sodium carboxylate in an N-alkyllactam in an amount of water of 1.2 to 2.4 moles with respect to mole of the alkali metal sulfide.

Those processes, however, present the disadvantages that the water content in the polymerization system needs to be set at a relatively low value within a narrow range in order to provide the polymer with a high production efficiency, which has such a high molecular weight as being highly valuable.

In usual cases, commercially available alkali metal sulfides and hydrosulfides contain a considerably large amount of water so that, if such compounds are employed as a raw material for polymerization as they are, the resulting polymers are not provided with a sufficiently high molecular weight, and a color is imparted to the resulting polymers.

For these reasons, the conventional processes require the alkali metal sulfides or hydrosulfides available usually as a hydrate to be dehydrated prior to polymerization, thereby removing water from the hydrous sulfides or hydrosulfides to reduce a large water content to a relatively low value within a narrow range. Accordingly, the conventional processes require at least two steps, such as dehydration and polycondensation steps. The provision of a dehydration step presents the following disadvantages. The dehydration step is usually carried out by means of a distillation in the presence of a polar solvent. However, a stainless steel reactor may get eroded and impurities may be dissolved from the inner wall of the reactor into the polar solvent, thereby impairing the purity and whiteness of the resulting polyarylene sulfide, or transforming the polar solvent to be recovered.

Furthermore, Japanese Patent Publication (Kokai) No. 22,926/1984 discloses a process in which a dihalogen aromatic compound is reacted with a substantially anhydrous metal sulfide and a metal carbonate in the presence of a minute amount of water. This process, however, produces a large amount of by-products, such as oligomers, and provides polyarylene sulfides with an insufficiently high molecular weight. The yield of the resulting polymer is low. Furthermore, a long period of time is required for enlargement of the molecular weight of the resulting polymer, so that this process is industrially disadvantageous.

The processes disclosed in U.S. Pat. Nos. 3,919,177 and 4,116,947 and Japanese Patent Publication No. 12,240/-1977 further suffer the disadvantages that a recovery and re-use of the polymerization promoter, such as sodium acetate or lithium acetate, is difficult. In order to recover the polymerization promoter, the process becomes so complicated that the economy is worsened. If the polymerization promoter is discharged into a water sewage system, a pollution problem arises.

Furthermore, the process disclosed in U.S. Pat. No. 4,116,947 uses a polyhgalogen aromatic compound as a branching agent, thereby enlarging molecular weights of branched polyphenylene sulfides.

As other processes for enlarging a molecular weight of a polyarylene sulfide, Japanese Patent Publication (Kokoku) No. 7,332/1986 propose processes, however, they present the disadvantage that the resulting polymers are likely to get gelled so that they pose problems with manufacture and quality.

Furthermore, the process disclosed in U.S. Pat. No. 4,116,947 has the drawback that the organic polar solvent used is restricted to expensive solvents, such as the lactams.

For instance, U.S. Pat. No. 3,867,356 discloses a process in which a dihalogen aromatic compound is reacted with an alkali metal hydrosulfide in an organic amide compound as a solvent in the presence of an alkali metal aminocarboxylate as represented by the general formula:

$$R_2N(CR_2)_nCO_2M$$

where

R is a hydrogen atom, an alkyl group, a cycloalkyl group or an aryl group, or a combination thereof;

M is lithium, sodium, potassium, rubidium or cesium; and

n is an integer from 1 to 12.

This process, however, presents the problem that the resulting polyarylene sulfide is colored due to by-products created by the side reactions between the polyhalogen aromatic compound and the alkali metal aminocarboxylate, thereby not yielding polyarylene sulfides with a high degree of whiteness and with a high molecular weight.

U.S. Pat. No. 3,869,433 proposes a process in which an alkali metal hydroxide is present in the reaction of a polyhalogen aromatic compound with an alkali metal hydrosulfide in an organic amide compound as a solvent. In this process, however, the organic amide compound is decomposed so that the solvent cannot be recovered and reused. This is industrially disadvantageous. Furthermore, the resulting polyarylene sulfide is colored, whereby polyarylene sulfide with a high degree of whiteness is not made.

U.S. Pat. No. 3,876,591 discloses a process for preparing polyarylene sulfides, in which an alkali metal hydrosulfide is used at amounts by 0.8 to 1.5 times of the molar amount of a polyhalogen aromatic compound. This process produces a large quantity of oligomers as by-products, thereby lessening the yield of the polyarylene sulfide and not providing polyarylene sulfide with a high molecular weight.

These conventional processes have the drawbacks that polyarylene sulfides cannot be prepared which have sufficient melt properties and unique characteristics such as functional groups. Thus demands have been made to provide novel polyarylene sulfides and a process for the preparation thereof in order to satisfy demands for various and diversified properties.

SUMMARY OF THE INVENTION

Therefore, as one present invention has the object to provide a process for preparing a novel polyarylene sulfide, which allows polymerization with ease and stability to a comparably high molecular weight value over a broad range of a water content, which produces a polyarylene sulfide having a high degree of whiteness and a sufficiently high molecular weight in higher yields, which can recover a polymerization promoter or aid with ease and which permits the efficient production, in high yields, of a polyarylene sulfide which is sufficiently high in molecular weight and excellent in whiteness, even if a hydrate or an aqueous mixture of an alkali metal sulfide or hydrosulfide which is readily available on the market, such as an industrial grade hydrous sodium sulfide is used.

The present invention has another object to provide a process which can prepare a polyarylene sulfide with high efficiency and in high yields, which polyarylene sulfide is high in whiteness and different from the usual polymers in solubility and other properties.

The present invention has a further object to provide a polyarylene sulfide with a novel structure, which is high in whiteness and different from the usual polymers in solubility and other properties.

In order to achieve these objects, the present invention consists of a process for preparing a polyarylene sulfide, comprising the step of contacting a dihalogen aromatic compound with a source of sulfur and a metallic salt of ω-hydroxycarboxylic acid in an organic polar solvent.

In order to achieve a further object, the present invention consists of a polyarylene sulfide containing a carbonyl group, which is insoluble in α-chloronaphthalene.

BRIEF DESCRIPTION OF THE DRAWING

The single FIGURE is an infrared absorption spectrum of one example of the compounds obtained by the process of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The process of and the polyarylene sulfide made according to, the present invention will be described.

The process according to the present invention involves contacting a sulfur source and a metallic salt of an ω-hydroxycarboxylic acid with the dihalogen aromatic compound reactant in an organic polar solvent.

The sulfur source may be selected from an alkali metal sulfide, an alkali metal hydrosulfide, and hydrogen sulfide.

The alkali metal sulfide may include, for example, sodium sulfide, lithium sulfide, potassium sulfide, rubidium sulfide and cesium sulfide. Preferred are lithium sulfide and sodium sulfide. The alkali metal sulfide may be used singly or in combination thereof.

The alkali metal hydrosulfide may include, for example, lithium hydrosulfide (LiHS), sodium hydrosulfide (NaHS), rubidium hydrosulfide (RbHS), potassium hydrosulfide (KHS), and cesium hydrosulfide (CsHS). Sodium hydrosulfide and rubidium hydrosulfide are preferred, and sodium hydrosulfide is more preferred. The alkali metal hydrosulfide may be used singly or in combination thereof.

The alkali metal sulfides and hydrosulfides may be used in the form of an anhydride form or in the form of a commercially available or industrial grade hydrate, hydrous material, aqueous solution, or aqueous mixture. When they are used in the form of the hydrate, hydrous material, aqueous solution or aqueous mixture, they may be dehydrated prior to polymerization if they contain a water content so large as not to set the water content of the polymerization system within the range as will be described hereinabove or they may be employed as they are without dehydration if they contain water in such an amount that the water content in the polymerization system can be set within the range.

It is preferred that the alkali metal hydrosulfide is employed with a base. The base may be any acid receptor which can convert the alkali metal hydrosulfide into the alkali metal sulfide or effectively neutralize or receive a hydrogen halide producible by condensation of the alkali metal hydrosulfide with the dihalogen aro-

matic compound and which does not adversely affect the object of the present invention. The base may be of an inorganic type and of an organic type. The inorganic salt may preferably include, for example, an alkali metal hydroxide, such as lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, and cesium hydroxide. Lithium hydroxide and sodium hydroxide are preferred, and sodium hydroxide is more preferred. The base may be used usually at least in an amount of an equimolar equivalent at the maximum per equivalent (mole) of the alkali metal hydrosulfide. The base may be used singly or in combination thereof.

As the sulfur source there may be used hydrogen sulfide together with or in place of the alkali metal hydrosulfide. It is preferred to use hydrogen sulfide which has been sufficiently purified. When hydrogen sulfide is used in place of the alkali metal hydrosulfide, it is preferred to use a base as in the case where the alkali metal hydrosulfide is used. The base to be used together with hydrogen sulfide may be the same as that used with the alkali metal hydrosulfide, as well as the kind, preferred kind, and amount of the base may the same as it is employed with the alkali metal hydrosulfide.

As the metallic salt of the ω-hydroxycarboxylic acid may be employed various kinds, and generally the metallic salt of the ω-hydroxycarboxyl acid may be represented by the following general formula:

$$HO-(CH_2)_nCOOM$$

wherein

M is an alkali metal; and n is an integer of 2 to 8, preferably 3 to 5.

The alkali metal represented by the symbol M may include, for example, lithium, sodium, potassium, rubidium or cesium. Lithium and sodium are preferred, and sodium is more preferred.

The metallic salt of the ω-hydroxycarboxylic acid as represented by the above general formula may include, for example, lithium 4-hydroxybutyrate, sodium 4-hydroxybutyrate, potassium 4-hydroxybutyrate, rubidium 4-hydroxybutyrate, cesium 4-hydroxybutyrate, lithium 5-hydroxyvalerate, sodium 5-hydroxyvalerate, potassium 5-hydroxyvalerate, rubidium 5-hydroxyvalerate, cesium 5-hydroxyvalerate, lithium 6-hydroxycaprylate, sodium 6-hydroxycaprylate, potassium 6-hydroxycaprylate, rubidium 6-hydroxycaprylate, cesium 6-hydroxycaprylate, and so on. Preferred is sodium 4-hydroxybutyrate. The metallic salt of the ω-hydroxycarboxylic acid may be used singly or in combination thereof. The metallic salt of the ω-hydroxycarboxylic acid may be used in the form of an anhydride, a hydrate or an aqueous solution. When it is used in the form of a hydrate or an aqueous solution, it is dehydrated prior to polymerization in the manner as will be described hereinbelow as in the case where the alkali metal hydrosulfides or sulfides are used in the form of a hydrate or an aqueous solution.

The dihalogen aromatic compound to be used for the process according to the present invention may be any conventional one and may include, for example, a dihalogen aromatic compound such as m-dihalobenzene and p-dihalo-benzene: an alkyl-substituted dihalobenzene such as 2,3-dihalotoluene, 2,5-dihalotoluene, 2,6-dihalotoluene, 3,4-dihalotoluene, 2,5-dihaloxylene, 1-ethyl-2,5-dihalobenzene, 1,2,4,5-tetramethyl-3,6-dihalobenzene and 1-n-hexyl-2,5-dihalobenzene; a cycloalkyl-substituted dihalobenzene such as 1-cyclohexyl-2,5-dihalobenzene; an aryl-substituted dihalobenzene such as 1-phenyl-2,5-dihalobenzene, 1-benzyl-2,5-dihalobenzene and 1-p-toluyl-2,5-dihalobenzene; a dihalobiphenyl such as 4,4'-dihalobiphenyl; and a dihalonaphthalene such as 1,4-dihalonaphthalene, 1,6-dihalonaphthalene, and 2,6-dihalonaphthalene. The two halogen atoms in the dihalogen aromatic compounds may be the same or different from each other and may be those replaced, for example, by fluorine, chlorine, bromine or iodine. Dihalobenzenes are preferred, and p-dichlorobenzene and p-dichlorobenzene containing 20 mol% or lower of m-dichlorobenzene are particularly preferred.

As the organic polar solvent may be used a an aprotic polar solvent such as an organic amide compound, a lactam compound, a urea compound, and a cyclic organophosphorus compound.

The amide compound may include, for example, a formamide such as N,N-dimethylformamide, an acetamide such as N,N-dimethylacetamide, N,N-diethylacetamide and N,N-dipropylacetamide, and an amide such as N,N-dimethylbenzamide, and the like.

The lactam compound may include, for example, a caprolactam such as caprolactatm, N-methylcaprolactam, N-ethylcaprolactam, N-n-propylcaprolactam, N-isopropylcaprolactam, N-n-butylcaprolactam, N-isobutylcaprolactam and N-cyclohexylcaprolactam, a pyrrolidone such as N-methyl-2-pyrrolidone, N-ethyl-2-pyrrolidone, N-n-propyl-2-pyrrolidone, N-isopropyl-2-pyrrolidone, N-n-butyl-2-pyrrolidone, N-isobutyl-2-pyrrolidone, N-cyclohexyl-2-pyrrolidone, N-methyl-3-methyl-2-pyrrolidone, N-ethyl-3-methyl-2-pyrrolidone and N-methyl-3,4,5-trimethyl-2-pyrrolidone, a piperidone such as N-methyl-2-piperidone, N-ethyl-2-piperidone, N-propyl-2-piperidone, N-isopropyl-2-piperidone, N-methyl-6-methyl-2-piperidone, N-methyl-3-ethyl-2-piperidone, and the like.

The urea compound may include, for example, tetramethyl urea, N,N'-dimethylethylene urea, and N,N'-dimethylpropylene urea.

As the cyclic organophosphorus compound may be used, for example, 1-methyl-1-oxosulphorane, 1-ethyl-1-oxosulphorane, 1-phenyl-1-oxosulphorane, 1-methyl-1-oxophosphorane, 1-n-propyl-1-oxophosphorane, 1-phenyl-1-oxophosphorane, and the like.

Among the organic polar solvents, the amide compound or lactam may be conveniently used. Preferred are an N-alkyllactam and an N-alkylpyrrolidone. More preferred is N-methylpyrrolidone. The organic polar solvent may be used singly or in combination thereof.

In accordance with the present invention, the polymerization may be carried out by contacting the sulfur source with the dihalogen aromatic compound and the metallic salt of the ω-hydroxycarboxylic acid in appropriate ratios in the organic polar solvent. In this reaction, the metallic salt of the ω-hydroxycarboxylic acid serves as a polymerization promoter or aid.

The metallic salt of the ω-hydroxycarboxylic acid may be used in an amount ranging generally from 0.02 to 2 moles, preferably from 0.05 to 1 mole, with respect to mole of the alkali metal sulfide and/or the alkali metal hydrosulfide. If the amount is below the lower limit, a polymerization rate becomes so slow that a molecular weight of the resulting polymer cannot be enlarged. If the amount exceeds the upper limit, a polymerization rate cannot be accelerated or a molecular weight of the polymer cannot be enlarged as such a large amount of the metallic salt of the ω-hydroxycar-

boxylic acid is used, as well as a cost of the polymerization promoter is raised.

It is interesting to note herein that the metallic salt of the ω-hydroxycarboxylic acid is incorporated into the polymer, on top of the serving as the polymerization promoter, thereby serving, too, to yield the novel polyarylene sulfide containing a carbonyl group, which is insoluble in α-chloronaphthalene.

In order to provide the novel polyarylene sulfide according to the present invention, the metallic salt of the ω-hydroxycarboxylic acid may be used in an amount ranging generally from 0.8 to 2.0 moles, preferably from 1 to 2 moles, with respect to mole of the alkali metal sulfide and/or hydrosulfide, or it may be used in an amount generally from 1.6 to 4 moles, preferably from 2 to 4 moles, with respect to mole of hydrogen sulfide.

The dihalogen aromatic compound may be used in an amount ranging generally from 0.75 to 2.0 moles, preferably from 0.90 to 1.2 moles, with respect to mole of the sulfur source. The use of the dihalogen aromatic compound in the amount below the lower limit may produce by-products, while the use thereof above the upper limit may reduce a molecular weight of the resulting polyarylene sulfide.

The amount of the organic polar solvent is not restricted to a particular range as long as it is sufficient to proceed with the reaction in a homogeneous manner. It may be conveniently in the range from 0.1 to 10 times of the total weight of the components consisting of the sulfur source, dihalogen aromatic compound, the metallic salt of the ω-hydroxycarboxylic acid, as well as the additives and other components to be added as desired. If the solvent is used in the amount below the lower limit, the reaction does not proceed to a sufficient extent. If the amount of the solvent exceeds the upper limit, a volume efficiency is worsened, thereby reducing a productivity.

In the polymerization system, water may be contained at a rate ranging from approximately 0.2 to 6 moles per mole of the sulfur source. If the water content is below the lower limit, a polymerization rate becomes so slow that a molecular weight of the polymer cannot be enlarged. The water content above the upper limit in the polymerization system may cause by-production, or enlargement of a molecular weight of the polymer may become insufficient. Furthermore, a pressure may in some cases be elevated to a remarkably high level during polymerization.

In the conventional processes for the production of the polyarylene sulfides, commercially available or industrial grade alkali metal sulfides or hydrosulfides are used for polymerization usually after dehydration. It is to be noted, however, that the process of the present invention does not require dehydration prior to polymerization and the reaction may proceed without intervention even if such alkali metal sulfides or hydrosulfides are used and a large quantity of water is contained in the polymerization system. Furthermore, this process can provide polyarylene sulfides having a high degree of whiteness and a relatively high molecular weight. This is deserved to draw attention.

Accordingly, the process according to the present invention is a simpler yet more useful process because a dehydration step can be omitted even if commercially available or industrial grade alkali metal sufides or hydrosulfide in the form of a hydrate or an aqueous mixture with a relatively large water content would be used as they are.

It is to be noted, however, that water may be added to the polymerization system together with or separately from the sulfur source such as the alkali metal sulfide or hydrosulfide, or in combination thereof.

As the step of adjusting the water content in the polymerization system, there may be used the step of merely admixing the components to be used without the use of dehydration prior to polymerization, by adding water if required, or the step of dehydrating the components prior to polymerization. In other words, as long as the water content in the polymerization system can be adjusted within the range as have been specified hereinabove, the dehydration step may not be required to be carried out prior to polymerization, although the dehydration step may be carried out prior to the polymerization step, if required.

In accordance with the process of the present invention in which the sulfur source is cotacted with the dihalogen aromatic compound and the metallic salt of the ω-hydroxycarboxylic acid, various additives may be optionally added to the polymerization system. The additives may include, for example, another polymerization promoter or aid, a branching agent such as an active hydrogen-containing halogen aromatic compound, a polyhalogen aromatic compound, and a halogen aromatic nitro compound, an end-capped agent such as a monohalogen aromatic compound or an active hydrogen-containing compound, a reducing agent, an inert organic solvent, and so on.

Such another polymerization promotor or aid may include, for example, an alkali metal halide, an alkali metal carboxylate, an alkali metal carbonate, an alkali metal borate, and so on.

The alkali metal halide may include, for example, a fluoride, chloride, bromide and iodide of an alkali metal such as lithium, sodium, potassium, rubidium, and cesium. More specifically, the alkali metal halide may include, for example, lithium fluoride, sodium fluoride, potassium fluoride, lithium chloride, sodium chloride, potassium chloride, rubidium chloride, cesium chloride, lithium bromide, sodium bromide, cesium bromide, lithium iodide, sodium iodide, potassium iodide, cesium iodide, and the like. Preferred is lithium chloride.

The alkali metal carboxylate may include, for example, an alkali metal salt, such as lithium, sodium, potassium, and cesium, of a carboxylic acid including a saturated aliphatic carboxylic acid such as formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, hexanoic acid, octanoic acid, and stearic acid; an aromatic carboxylic acid such as benzoic acid; a saturated aliphatic dicarboxylic acid such as oxalic acid and malonic acid; an aliphatic unsaturated dicarboxylic acid such as fumaric acid; an aromatic dicarboxylic acid such as phthalic acid; and an aliphatic oxy acid such as tartaric acid. More particularly, the alkali metal carboxylates may include, for example, lithium formate, sodium formate, lithium acetate, sodium acetate, potassium acetate, cesium acetate, lithium propionate, sodium propionate, lithium butyrate, sodium butyrate, lithium isobutyrate, sodium isobutyrate, lithium valerate, sodium valerate, lithium hexanoate, sodium hexanoate, lithium octanoate, sodium octanoate, lithium stearate, sodium stearate, lithium benzoate, sodium benzoate, potassium benzoate, lithium oxalate, sodium oxalate, lithium malonate, sodium malonate, lithium fumarate, sodium fumarate, lithium phthalate, sodium phthal-

ate, lithium tartrate, and sodium tartrate. Preferred are lithium acetate, sodium acetate, and lithium benzoate.

The alkali metal carbonate may include, for example, lithium carbonate, sodium carbonate, potassium carbonate, rubidium carbonate and cesium carbonate. Lithium carbonate and sodium carbonate is preferred, and lithium carbonate is more preferred.

The alkali metal borate may include, for example, lithium borate, sodium borate, potassium borate, and cesium borate. Lithium and sodium borates are preferred, and lithium borate is preferred.

As the active hydrogen-containing halogen aromatic compound as one of the branching agent may be employed a halogen aromatic compound with an active hydrogen-containing group or groups, such as an amino group, mercapto group, hydroxyl group or the like. More specifically, the active hydrogen-containing halogen aromatic compound may include, for example, a dihaloaniline such as 2,6-dichloroaniline, 2,5-dichloroaniline, 2,4-dichloroaniline and 2,3-dichloroaniline, a trihaloaniline such as 2,3,4-trichloroaniline, 2,3,5-trichloroaniline, 2,4,6-trichloroaniline and 3,4,5-trichloroaniline, an amino-containing halogen aromatic compound including a dihaloaminodiphenyl ether such as 2,2'-diamino-4,4'-dichlorodiphenyl ether and 2,4'-diamino-2',4-dichlorodiphenyl ether, and a mixture thereof. Furthermore, those in which the amino group is replaced by the other functional group such as the thiol group or the hydroxyl group may also be used. There may also be used an active hydrogen-containing halogen aromatic compound, in which a hydrogen atom or atoms joined to the carbon atom or atoms forming its aromatic ring is or are replaced by another inert group or groups such as a carbohydryl group, i.e., an alkyl group. Among the active hydrogen-containing halogen aromatic compounds, the active hydrogen-containing dihalogen aromatic compound is preferred. Dichloroaniline is more preferred.

The polyhalogen aromatic compound as one of the branching agents may include, for example, a trihalobenzene such as 1,2,4-trichlorobenzene and 1,3,5-trichlorobenzene and a trihalonaphthalene such as 1,4,6-trichloronaphthalene. Particularly, 1,2,4-trichlorobenzene and 1,3,5-trichlorobenzene are preferred.

The halogen aromatic nitro compound may include, for example, a mono- or dihalonitrobenzene such as 2,4-dinitrochlorobenzene or 2,5-dichloronitrobenzene, a dihalonitrodiphenyl ether such as 2-nitro-4,4'-dichlorodiphenyl ether, a dihalonitrodiphenyl sulfone such as 3,3'-dinitro-4,4'-dichlorodiphenyl sulfone, a mono- or di-halonitropyridine such as 2,5-dichloro-3-nitropyridine or 2-chloro-3,5-dinitropyridine, or a dihalonitronaphthalene.

The use of the branching agent, such as the active hydrogen-containing halogen aromatic compound, the polyhalogen aromatic compound, and the halogen aromatic nitro compound may serve as increasing branches of the resulting polymer, thereby further enlarging a molecular weight thereof, or as reducing a salt content, thereby further improving various properties of the resulting polymer.

The amount of the branching agent may be in the range generally from 0.0002 to 0.05 mole, preferably from 0.002 to 0.03 mole, with respect to mole of the dihalogen aromatic compound.

The monohalogen aromatic compound to be added as an end-capped agent may include, for example, chlorobenzene, bromobenzene, α-bromobenzene, α-chlorotoluene, o-chlorotolucne, m-chlorotoluene, p-chlorotoluene,α-bromotoluene, o-bromotoluene, m-bromotoluene, and p-bromotoluene.

The active hydrogen-containing compound may include, for example, a thiophenol, phenol, aniline, and so on.

As a branching agent or an end-capped agent, a compound having three or more than three reactive halogen atoms, such as cyanuric chloride, may also be used.

The branching agent or the end-capped agent may be used singly or in combination thereof.

The reducing agent may include, for example, a hydrazine, metal hydride, alkali metal formate, and sulfur. The metal hydride is preferred, as well as sodium borohydride and calcium hydride are more preferred.

The inert solvent may include, for example, a hydrocarbon such as benzene, toluene, xylene, biphenyl, terphenyl, naphthalene, or anthracene, an ether such as diphenyl ether, p-diphenoxybenzene, polyethylene glycol, or dioxane, or the like. A high-boiling-point inert organic solvent is preferred.

In accordance with the process of the present invention, the components such as the sulfur source, the metallic salt of the ω-hydroxycarboxylic acid, and the dihalogen aromatic compound may be added in any order as long as they can be polymerized in the organic polar solvent. A preferred feature will be described hereinafter.

In other words, the sulfur source, the dihalogen aromatic compound, and the metallic salt of the ω-hydroxycarboxylic acid are contacted with each other in the organic polar solvent such as the organic amide compound.

It is preferred that a mixture of the sulfur source and the dihalogen aromatic compound with the organic polar solvent is first dehydrated and the resulting dehydrated mixture is then contacted with the metallic salt of the ω-hydroxycarboxylic acid.

The dehydration can be carried out by means of a distillation. It is preferred that the dehydration is carried out, for example, at a temperature of 150° to 202° C. in an inert gas, such as in a stream of nitrogen. however, the dehydration may be effected by heating under reduced pressures. A dehydrating agent such as calcium oxide or calcium chloride may be employed.

The polymerization may be carried out at a temperature ranging generally from 180° to 330° C., preferably from 210° to 290° C. Temperatures below the lower limit makes a reaction rate so slow that it is not practically applicable, while temperatures above the upper limit causes by-production and degrades or decomposes the resulting polymer, thereby imparting a color or causing gellation.

Although a reaction time may vary with kinds and amounts of the various components and polymerization promoter or aid, or a reaction temperature, it may range generally within 24 hours, preferably from 1 to 24 hours.

The polycondensation may be carried out in an ambient atmosphere of an inert gas such as nitrogen, argon or carbon dioxide.

A reaction pressure is not restricted to a particular range and may be generally up to 50 kg/cm^2, (absolute pressure) from the self pressure of the polycondensation system such as the solvent. The polycondensation may be a one-stage reaction in which the reaction is carried out at a stationary temperature or a multiple-stage reaction in which the reaction temperature is elevated in a

stepwise manner or in a continuously ascending manner.

After completion of the polymerization, the resulting polyarylene sulfide may be isolated by separating it directly from a reactor by a standard method such as filtration or centrifugal separation or by separating it from the reaction mixture after addition of a flocculating agent such as water and/or a dilute acid.

The polymer separated is then washed usually with water, methanol, methylene chloride, chloroform, acetone, benzene, toluene, or the like, to remove the alkali metal halide, alkali metal hydrosulfide, solvent and the like adhering to the polymer. The resulting polymer may be effectively recovered by removing the solvent from the reaction mixture without separation therefrom and by washing the residue in the same manner as above. The solvent recovered may be reused.

If the sulfur source is used in an excessive amount, the reaction mixture (a mother liquor) separated and/or washings used may be adjusted to an acidic range and distilled to thereby recover the excessive amount of the metallic salt of the ω-hydroxycarboxylic acid as a lactone which, in turn, is conveniently purified and then treated with an alkali metal hydroxide, thereby converting it to the corresponding metallic salt of the carboxylic acid. This may be re-used.

The process according to the present invention provides the polyarylene sulfides having a high degree of whiteness in high yields with a high production efficiency as well as with ease and stability.

The polyarylene sulfides according to the present invention presents the characteristics that they contains a carbonyl group, which is prepared by using the metallic salt of the ω-hydroxycarboxylic acid, and that they are insoluble in α-chloronaphthalene which is used for measuring an intrinsic viscosity of the polyarylene sulfides. Accordingly, they can be utilized as molding materials and, if necessary, may be subjected to desalting treatment in order to reduce a content of a salt such as sodium chloride in the polymer.

In molding the polyarylene sulfides according to the present invention, for example, other polymers, pigments, fillers such as graphite, talc, calcium carbonate, mica, carbon black, glass powder, quartz powder, glass fibers, carbon fibers, stabilizers, lubricants may be added.

The polyarylene sulfides according to the present invention may be used as matrix resins for various molded products and composite materials, and they can be molded into films, sheets, fibers, and various articles and items and can be conveniently used for mechanical, electrical and electronic parts.

The present invention will be described by way of examples with reference to comparative examples.

Synthesis of Sodium 4-Hydroxybutyrate:

A solution was prepared by dissolving 50.5 g (1.26 moles) of sodium hydroxide in 300 ml of purified water.

To this sodium hydroxide aqueous solution was dropwise added 172 g (2 moles) of γ-butyrolactone gradually at room temperature. After completion of the dropwise addition, the mixture was elevated to 100° C. and the reaction was carried out for 1 hour. After completion of the reaction, approximately 800 ml of water was removed off, leaving crude materials in a white color. The crude materials are washed twice with 1 liter of acetone and then dried in vacuo, yielding 190.81 g of white powdery product.

The white powdery product was confirmed that it was sodium 4-hydroxycarboxybuyrate as a result of infrared absorption spectrum as follows:

$$\nu_{CO_2Na} = 1,500 - 1,600\ cm^{-1}$$

$$\nu_{OH} = 3,200 - 3,400\ cm^{-1}$$

The melting point [Tm] of the white powdery product was Tm = 185° C.

EXAMPLE 1

A 1-liter autoclave was charged with 70.59 g (0.543 mole) of commercially available sodium sulfide of industrial grade (water content: approximately 40% by weight), 79.83 g (0.543 mole) of p-dichlorobenzene, 6.84 g (0.543 mole) of sodium 4-hydroxybutyrate, and 304 ml of N-methylpyrrolidone, and replenished with nitrogen gas. The autoclave was closed and elevated to 260° C. at which the polycondensation was carried out for 3 hours.

After completion of the reaction, the temperature within the autoclave was cooled down to room temperature, and the reaction mixture was washed with water and acetone in conventional manner, leaving a solid material.

The solid material was then dried by heating at 100° C. under highly reduced pressures over the period of 20 hours, yielding 49.85 g (85%) of polyphenylene sulfide in a white color.

This product was found to have an inherent viscosity [η_{inn}] of 0.17 when measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 206° C.

It was further found that N-methylpyrrolidone recovered was little colorless and no rust was formed on the autoclave.

EXAMPLE 2

The procedures of Example 1 were followed with the exception that, in place of hydrous sodium sulfide, 63.35 g (0.543 mole) of commercially available sodium hydrosulfide of industrial grade (NaHS; water content: about 52% by weight), and 21.72 g (0.543 mole) of sodium hydroxide in a pellet form were used, yielding 48.67 g (83%) of polyphenylene sulfide in a white color.

This product was found to have an inherent viscosity [η_{inn}] of 0.16 when measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 200° C.

It was further found that N-methylpyrrolidone recovered was in a pale yellow and no rust was formed on the autoclave.

EXAMPLE 3

The procedures of Example 1 were followed with the exception that the amount of sodium 4-hydroxybutyrate was changed to 20.52 g (0.163 mole) and 12.7 g of water was added, yielding 51.61 g (88%) of polyphenylene sulfide in a white color.

This product was found to have an inherent viscosity [η_{inn}] of 0.15 when measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 200° C.

COMPARATIVE EXAMPLE 1

The procedures of Example 1 were followed with the exception that sodium 4-hydroxybutyrate was not used, yielding 53.95 g (92%) of polyphenylene sulfide in a pale brown color. This product was found to have an inherent viscosity [η_{inn}] of 0.11.

As is apparent from the above, the polymer obtained in the Comparative Example 1 has a molecular weight lower than those obtained in Examples 1 to 3 and it was poor in a color tone.

COMPARATIVE EXAMPLE 2

The procedures of Example 1 were followed with the exception that, in place of sodium 4-hydroxybutyrate, 4.45 g (0.0543 mole) of sodium acetate was used, yielding 49.26 g (84%) of polyphenylene sulfide in a greyish white color. This product was found to have an inherent viscosity [η_{inn}] of 0.15.

EXAMPLE 4

The procedures of Example 1 were followed with the exception that 0.269 g of 1,2,4-trichlorobenzene was additionaly used, yielding 49.85 g (85%) of polyphenylene sulfide. This product was found to have an inherent viscosity [η_{inn}] of 0.19.

EXAMPLE 5

A 1-liter autoclave was charged with a solution of 42.7 g (0.543 mole) of sodium sulfide (concentration: 71.3%) and 68.47 g (0.543 mole) sodium 4-hydroxybutyrate in 200 ml of water, and 297 ml of N-methylpyrrolidone. The autoclave was elevated to 154° C.in a stream of nitrogen gas and 295 ml of the mixture was removed from the autoclave. Gases removed was found to contain 0.01 mole of hydrogen sulfide gas.

The autoclave was then cooled down to 100° C. and 102 ml of a solution of 78.63 g (0.533 mole) of p-dichlorobenzene in 102 ml of N-methylpyrrolidone. The mixture was then reacted at 260° C. for 3 hours.

After completion of the reaction, the autoclave was opened and 1 liter of water was poured onto the reaction mixture, and the precipitated material was washed twice with 1 liter of water and once with 1 liter of acetone. The material was then dried by heating at 100° C. in vacuo over the period of 20 hours, yielding 41.5 g (72%, based on p-dichlorobenzene) of a polymer in a white color. This product was found to be insoluble when an inherent viscosity [η_{inn}] of 0.17 was measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 206° C. Its melting point [Tm] was Tm=278° C.

It was further found that the reaction mixture recovered was colored in pale yellow, and a small amount of scars was formed on the autoclave.

An infrared absorption spectrum analysis has revealed that this product had an absorption peak at 1,700 cm^{-1} based on the carbonyl group.

An infrared absorption spectrum is shown in FIGURE.

EXAMPLE 6

The procedures of Example 5 were followed with the exception that the amount of sodium 4-hydroxybutyrate was Changed from 68.47 g (0.543 mole) to 89.01 g (1.300 moles), yielding 42.4 g (73% based on p-dichlorobenzene) of polyphenylene sulfide. This product was found to be insoluble when an inherent viscosity [η_{inn}] was measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 206° C. Its melting point [Tm]was Tm=277° C.

The polymer was found to be the same as obtained in Example 5 as a result of observation for infrared absorption spectrum.

EXAMPLE 7

The procedures of Example 5 were followed with the exception that, in place of 68 47 g (0.543 mole) of sodium 4-hydroxybutyrate, 83.6 g (0.543 mole) of sodium 6-hydroxyhexanoate was used, yielding 38 05 g (66% based on p-dichlorobenzene) of a polymer.

This product was found to be insoluble when an inherent viscosity [η_{inn}] was measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 206° C. Its melting point [Tm] was 275° C.

COMPARATIVE EXAMPLE 3

The procedures of Example 5 were followed with the exception that sodium 4-hydroxybutyrate was not used and an additional amount of 68.07 g of p-dichlorobenzene was added in order to allow the amount of p-dichlorobenzene to become equimolar with respect to the mole of sulfur remaining in the autoclave because the amount of hydrogen sulfide gas contained in the discharged gases was as high as 0.08 mole at the time of dehydration, yielding 13.5 g (27% based on p-dichlorobenzene) of a polymer. This product was found to have an inherent viscosity [η_{inn}]of 0.03 when measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 206° C. Its melting point [Tm] was Tm=232° C.

It was further found that the solution after the reaction was colored in dark brown and a malodor was emitted.

COMPARATIVE EXAMPLE 4

The procedures of Example 5 were followed with the exception that, in place of 68.47 g (0.543 mole) of sodium 4-hydroxybutyrate, 21.7 g (0.543 mole) of sodium hydroxide was used, yielding 51.6 g (88% based on p-dichlorobenzene) of a polymer.

This product was found to have an inherent viscosity [θ_{inn}] of 0.10 when measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 206° C.

The solution after the reaction was colored in dark brown.

COMPARATIVE EXAMPLE 5

The procedures of Example 5 were followed with the exception that, in place of sodium 4-hydroxybutyrate, sodium N-methyaminobutyrate was used, yielding 48.2 g (82% based on p-dichlorobenzene) of a polymer. This product was found to have an inherent viscosity [θ_{inn}] of 0.14 when measured in 1-chloronaphthalene at a concentration of 0.4 g/dl at a temperature of 206° C.

The solution after the reaction was colored in dark brown. It was found that an additive of p-dichlorobenzene and sodium N-methylaminobutyrate was detected in the reaction mixture in the amount of 3,000 ppm so that it was assumed the additive was added to the polymer terminal and p-dichlorobenzene was lost in a large amount.

As have been described hereinabove, the present invention presents the advantages.

The use of the metallic salt of ω-hydrocarboxylic acid as a polymerization promoter or assistant permits a stable polymerization even if a large quantity of water is contained in the reaction system, yielding a polyarylene sulfide having a sufficiently high molecular weight with high efficiency.

The process permits an easy recovery of the polymerization promoter or aid.

The process also enables the use of alkali metal sulfides or hydrosulfides in the form of a hydrate or an aqueous mixture which is readily available, such as commercially available or industrial grade hydrous sodium sulfide, as they are without dehydration.

The process is a useful process for preparing polyarylene sulfides with a high efficiency in high yields, which have excellent properties such as a sufficiently high molecular weight and a high degree of whiteness.

The use of the metallic salt of ω-hydrocarboxylic acid at a particular ratio can provide a novel polyarylene sulfide in high yields without causing any side reactions, so that the product is not colored with any by-products, thus yielding the product having a high degree of whiteness. The metallic salt of ω-hydrocarboxylic acid used can be recovered as a lactone for re-use. The polyarylene sulfide is provided with a carbonyl group, so that it is industrially useful.

What is claimed is:

1. A process for preparing a polyarylene sulfide comprising the step of contacting a sulfur source with a metallic salt of ω-hydroxycarboxylic acid of the general formula:

$$HO-(CH_2)_n-COOM$$

wherein M is an alkali metal and n is an integer of 2 to 8, and a dihalogen compound in an organic polar solvent.

2. A process as claimed in claim 1, wherein:
the sulfur source is an alkali metal sulfide, an alkali metal hydrosulfide or hydrogen sulfide.

3. A process as claimed in claim 1, wherein:
the sulfur source is sodium sulfide or sodium hydrosulfide.

4. A process as claimed in claim 1, wherein:
the metallic salt of ω-hydroxycarboxylic acid is sodium 4-hydroxybutyrate, sodium 5-hydroxyvalerate, or sodium 6-hydroxycaprylate.

5. A process as claimed in claim 1, wherein:
the organic polar solvent is a an aprotic organic solvent.

* * * * *

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Cellular Response to Transforming Growth Factor-β1 and Basic Fibroblast Growth Factor Depends on Release Kinetics and Extracellular Matrix Interactions*

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The extracellular matrix plays an important role in growth factor biology, serving as a potential platform for rapid growth factor mobilization or a sink for concentrated sequestration. We now demonstrate that when a growth factor binds reversibly to the matrix, its effects are augmented by this interaction, and when the factor is absorbed irreversibly to the extracellular matrix, it becomes sequestered. These findings call into question the notion that all growth factors are best presented to cells and tissues in a sustained and controlled fashion. In our studies, we examined basic fibroblast growth factor (bFGF) and transforming growth factor-β1 (TGF-β1) release kinetics from synthetically fabricated microsphere devices and naturally synthesized extracellular matrix. While the sustained release of bFGF was up to 3.0-fold more potent at increasing vascular endothelial and smooth muscle cell proliferation than bolus administration, the reverse was true for TGF-β1. A bolus of TGF-β1 inhibited vascular cells up to 3.8-fold more efficiently than the same amount of TGF-β1 if control-released. Both growth factors bound to the extracellular matrix, but only bFGF was released in a controlled fashion (2.8%/day). Contact with the extracellular matrix and subsequent release enhanced bFGF activity such that it was 86% more effective at increasing smooth muscle cell numbers than equal amounts of growth factor diluted from frozen stock. TGF-β1 remained tightly adherent. The small amount of TGF-β1 released from the extracellular matrix was ~30% less effective than bolus administration at inhibiting vascular endothelial and smooth muscle cell growth. Sustained growth factor release may be the preferable mode of administration, but only when a similar mode of metabolism is utilized endogenously.

The extracellular matrix is an integral part of growth factor biology. Many growth factors bind to this substrate, and it has been postulated that the matrix serves as a sequestration site from which growth factor stores can be concentrated for enhanced local action or released for heightened overall effect (1–7). Basic fibroblast growth factor (bFGF)[1] is a model compound in this regard. Endogenous bFGF has been found localized to heparan sulfate-rich basement membranes *in vivo* (8, 9). The binding of bFGF to heparan sulfate proteoglycans protects bFGF from proteolytic degradation and may provide a reservoir of active bFGF in the extracellular matrix that may be available over a prolonged time (7, 10–15). The controlled release of bFGF might be important for maintenance of intact tissues, while acute injury might induce a burst release of bFGF following extracellular matrix degradation and tissue destruction. Accordingly, it has been assumed that the maximum biologic effect of bFGF and related growth factors is observed only when control-released. Indeed, we (16–18) and others (19–21) have demonstrated profound biologic effects of bFGF when control-released to cells in culture or tissues *in vivo*.

These findings have been extended to matrix-binding growth factors, and it is now well documented that cells optimally respond to the controlled release of growth factors (18, 22, 23). The exceedingly low doses, rapid clearance, and denaturation of these compounds even further support the idea that controlled or sustained release is an absolute requirement. A wide range of techniques have been created to ensure controlled delivery. Yet, few have investigated the validity of the underlying assumption that the most effective delivery of all growth factors to target cells and tissues is in a slow and controlled fashion, rather than a single bolus administration. This paradigm may not be true. Cells may paradoxically respond least well to sustained growth factor release. Consequently, this study sought to understand how, when, and why cells of the vasculature respond to different modes of growth factor administration and to compare effects obtained with bFGF with those obtained with transforming growth factor-β1 (TGF-β1).

Reports of growth factor physiology often compare bFGF and TGF-β1, for together they possess the fullest range of biochemical and biophysical parameters at opposite ends of the spectrum of biologic effects (24, 25). They both have short *in vivo* half-lives of <30 min (26–28) and yet have profound and sustained mediation of angiogenesis (29–32), gene expression (33–35), and extracellular matrix accumulation (36–39). Since cellular events such as receptor kinetics or ligand trafficking alone cannot explain the prolonged biologic effects and because both growth factors are matrix-binding, this interaction may be responsible for their sustained effects. We found, however, that while the sustained release of bFGF was up to 3.0-fold more potent at increasing vascular endothelial and smooth muscle cell proliferation than bolus administration, the reverse was true for TGF-β1. A bolus of TGF-β1 inhibited vascular cells up

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[1] The abbreviations used are: bFGF, basic fibroblast growth factor; TGF-β1, transforming growth factor-β1; DMEM, Dulbecco's modified Eagle's medium; EVAc, ethylene-vinyl acetate copolymer; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

to 3.8-fold more efficiently than the same amount of TGF-β1 if control-released. Both growth factors bound to the extracellular matrix, but only bFGF was released in a controlled fashion. Contact with the extracellular matrix and subsequent release enhanced bFGF activity. bFGF released from the extracellular matrix was more effective at increasing vascular cell growth than equal amounts of growth factor diluted from frozen stock. TGF-β1 remained tightly adherent, and the small amount of the growth factor released from the extracellular matrix was less effective than bolus administration at inhibiting vascular cell growth. Sustained growth factor release may be the preferable mode of administration, but only when a similar mode of slow release metabolism is utilized endogenously. A more precise understanding of the relationship between the matrix and growth factors will not only aid in the design of formulations for therapeutics, but will also lead to an unveiling of a more definite pathophysiology of the diseases that growth factors govern.

EXPERIMENTAL PROCEDURES

Cell Isolation—Endothelial and smooth muscle cells were isolated from freshly excised aortas of 3–4-week-old calves (Area and Sons, Hopkinton, MA). Endothelial cells were harvested by incubating the luminal side of the aorta with a 1 mg/ml collagenase digestion (Sigma) for 9.5 min. The collagenase was discarded, and the cells were removed by gently washing the luminal side with a 0.5-ml aliquot of high antibiotic medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (200 units/ml), streptomycin (200 μg/ml), 2% Fungizone, glutamine (100 mM), and 10% calf serum (all from Life Technologies, Inc.). The freshly isolated endothelial cells were placed in a 60 × 15-mm sterile Petri dish containing a sterile glass coverslip with 2.0 ml of high antibiotic medium and allowed to grow at 37 °C in a humidified 5% CO_2, 95% air incubator.

To isolate smooth muscle cells, the collagenase-digested luminal side of the aorta was cut open longitudinally and exposed. The luminal surface was gently scored with a sterile blade into 0.5-cm^2 sections. With sterile forceps, the scored squares were peeled away and placed media side down onto a 60 × 15-mm Petri dish. A sterile glass coverslip was placed on top of the sections to secure the pieces of tissue in place, and high antibiotic medium was pipetted around the sections and incubated at 37 °C as described above. When smooth muscle cells migrated away from the sections, the tissues were gently lifted off and discarded, and the smooth muscle cells were allowed to proliferate. Assessment of successful isolation was determined by inspection for consistent cell-specific morphology and by immunohistologic staining for von Willebrand's factor with endothelial cell cultures and α-smooth muscle cell actin with smooth muscle cell cultures.

Cell Maintenance—Both cell lines were subcultured with 0.25% trypsin, 1 mM EDTA in Hanks' balanced salt solution (Life Technologies, Inc.) when the cells were subconfluent. The cells were maintained in 75-cm^2 culture flasks (Costar Corp.) in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), glutamine (100 mM), and 10% calf serum. Both cell lines were cultured up to passage 5. Cell numbers were determined by counting trypsinized cells with a ZF1 Coulter Counter.

Cell Proliferation Assays—The differential effects of bolus or control-released amounts of bFGF or TGF-β1 on the proliferation of sparsely seeded (4×10^3 cells/ml/well) endothelial and smooth muscle cells were examined in 2.5-cm cluster 12-well tissue culture-treated plates (Costar Corp.). Endothelial cells were plated in DMEM containing 10% calf serum and allowed to attach overnight. Smooth muscle cells were plated in DMEM containing 5% calf serum, allowed to attach overnight, and then growth-arrested by changing the medium to 0.05% calf serum for 3 days.

bFGF proliferation assays were performed by changing the culture medium of 24-h endothelial or growth-arrested smooth muscle cells to DMEM containing 1% calf serum. Alginate/heparin-Sepharose controlled release microspheres with and without bFGF were placed into the culture wells with sterile forceps, or equivalent amounts of concentrated bFGF were added directly to the culture medium as a single bolus. TGF-β1 assays were performed by replacing the culture medium of 24-h endothelial or growth-arrested smooth muscle cells with fresh DMEM containing 10% calf serum. Ethylene-vinyl acetate copolymer (EVAc)-bovine serum albumin (BSA)-TGF-β1 controlled release microspheres with and without TGF-β1 were placed into the culture wells with sterile forceps, or equivalent amounts of concentrated TGF-β1 were added directly to the culture medium as a single bolus. Microsphere or bolus additions constituted day 1 of the proliferation assay. For both assays, cells were allowed to grow for the indicated times, and cell number was determined by trypsinization followed by number assessment with a ZF1 Coulter Counter.

Alginate/Heparin-Sepharose Microsphere Preparation and Growth Factor Incorporation—Alginate/heparin-Sepharose microspheres were prepared according to methods previously described (18, 22). Briefly, a 1.8% solution of low molecular weight sodium alginate (Sigma) was prepared in water and sterilized by filtration. Heparin-Sepharose beads (CL-6B, Pharmacia Biotech Inc.) were sterilized under ultraviolet light and allowed to swell in sterilized water. After three washes in water, the beads were resuspended in water (1:1, v/v). The sodium alginate solution and heparin-Sepharose beads were mixed together at a final concentration of 33.3 mg/ml heparin-Sepharose and 1.2% (w/v) sodium alginate. This mixed suspension was dropped through a sterile Pasteur pipette into a 500-ml gently stirring solution of 1.5% $CaCl_2$. Upon entering the $CaCl_2$ solution, the alginate/heparin-Sepharose suspension immediately formed hard spherical pellets. These microspheres were mixed gently for 5 min to cure and were then allowed to incubate in the same solution for 10 min without stirring. The microspheres were washed three times in sterile water and stored at 4 °C in 150 mM NaCl and 1 mM $CaCl_2$ for up to 3 months.

bFGF incorporation into the alginate/heparin-Sepharose microspheres was performed by first placing the microspheres in sterile microcentrifuge tubes coated with 150 mM NaCl, 1 mM $CaCl_2$, and 0.5% gelatin. For each microsphere, a 1-μl aliquot of bFGF (100 μg/ml; R&D Systems, Minneapolis, MN) or Bolton-Hunter labeled human recombinant ^{125}I-bFGF (70 μCi/μg; DuPont NEN) was added along with 14 μl of 150 mM NaCl, 1 mM $CaCl_2$, and 0.15% gelatin. The tubes were gently agitated overnight at 4 °C. bFGF release studies were performed as described by Nugent *et al.* (18) using three alginate/heparin-Sepharose microspheres.

EVAc Microsphere Preparation and Growth Factor Incorporation—EVAc microspheres were prepared according to the methods of Sefton *et al.* (40). Briefly, EVAc (Elvax 40, DuPont) was washed thoroughly in water and ethanol and then dissolved in methylene chloride to yield a 10% (w/v) solution. BSA was weighed out to provide a 30% loading (BSA/EVAc, w/w) in glass scintillation vials and was dissolved in ~2 ml of Milli-Q water. TGF-β1 (R&D Systems) or Bolton-Hunter labeled human recombinant ^{125}I-TGF-β1 (120–180 μCi/μg; DuPont NEN) was added to this suspension to equal 3 ng of TGF-β1/microsphere. The solution was lyophilized to a dry powder and crushed fine, and dissolved 10% EVAc solution was then added to the powder. This mixture was then dropped using a Pasteur pipette into a glass beaker filled with 50 ml of cold ethanol (−40 °C) that was placed in a dry ice-ethanol bath. Hard spherical pellets formed immediately as the mixture entered the cold ethanol solution. After 5–10 min, the ethanol solution containing the EVAc microspheres was allowed to warm to room temperature. The ethanol was changed, and the microspheres were allowed to cure overnight at ambient temperature. The next day, the ethanol was decanted, and any residual ethanol was removed by lyophilization. After the removal of the ethanol, the microspheres were kept under sterile conditions at room temperature. TGF-β1 release kinetics were followed for 5–10 EVAc-BSA-^{125}I-TGF-β1 microspheres placed in 1 ml of PBS at room temperature with gentle shaking. At designated times, the PBS containing the released ^{125}I-TGF-β1 was removed and replaced with a fresh 1-ml PBS aliquot for 10 days. The amount of radioactivity was assessed using a Pharmacia Biotech scintillation counter.

Extracellular Matrix Incorporation and Release of Growth Factors—Endothelial or smooth muscle cells were plated at 1×10^4 cells/ml/well in 2.5-cm cluster 12-well tissue culture-treated plates using DMEM supplemented with glutamine, antibiotics, and 10% calf serum. Cells reached confluency in ~7 days. The confluent monolayers were washed one time in PBS (Ca^{2+}- and Mg^{2+}-free) and incubated with 1 ml/well extraction buffer (0.5% Triton X-100, 20 mM NH_4OH in Ca^{2+}- and Mg^{2+}-free PBS) for 5 min at room temperature to solubilize the cells. The extra buffer was removed, and the wells were washed five times with PBS (Ca^{2+}- and Mg^{2+}-free) to remove the solubilized cells, leaving extracellular matrix coating the bottom of the wells (41). The extracellular matrix-coated wells were covered with 1 ml of Ca^{2+}- and Mg^{2+}-free PBS and retained at 37 °C until continuing with subsequent procedures.

bFGF, TGF-β1, ^{125}I-bFGF, or ^{125}I-TGF-β1 was incorporated into the extracellular matrix by first incubating the matrix with 1 ml of blocking buffer (DMEM, 50 mM Hepes, pH 7.4, containing 0.05% gelatin) for 10 min at room temperature. The blocking buffer was discarded, and

another 1-ml aliquot of the blocking buffer was placed on the matrix. Growth factor at 1–10 ng/ml concentrations was added and then incubated at 4 °C for 2 h. The buffer solution was removed, and the growth factor-incorporated extracellular matrix was washed three times with fresh blocking buffer at 4 °C. To determine the amount of incorporated growth factor, the matrix from the ^{125}I-bFGF or ^{125}I-TGF-β1 incorporation was removed by placing 1 ml of 1 N NaOH in the well and incubating the well for 5 min at room temperature. The NaOH solution containing the suspended extracellular matrix with incorporated ^{125}I-growth factor was counted using a Pharmacia Biotech 1272 CliniGamma γ-counter.

In other identical plates, the release of ^{125}I-bFGF or ^{125}I-TGF-β1 from the extracellular matrix was monitored. Wells were covered with 1 ml of release buffer (DMEM, 50 mM Hepes, pH 7.4, containing 0.15% gelatin) at 37 °C. At designated times, the buffer now containing released growth factors was removed and replaced with fresh 1-ml aliquots of release buffer. The amount of radioactivity in each aliquot was assessed by γ-counting.

Nonradiolabeled bFGF or TGF-β1 (1–10 ng/ml) in blocking buffer was exposed to the extracellular matrix for 2 h at 4 °C. The buffer solution was removed, and the extracellular matrix was washed three times with fresh blocking buffer at 4 °C. Fresh release buffer was placed on the wells, retrieved after 1 h at 37 °C, replaced with a fresh 1-ml aliquot, and then removed after the second hour. The second-hour aliquot represented growth factor bound and subsequently released from the extracellular matrix. This aliquot was transferred onto PBS-washed endothelial or smooth muscle cells plated at 4×10^3 cells/ml/well. Cells were allowed to grow for the indicated times, and cell number was determined by trypsinization followed by number assessment with a ZF1 Coulter Counter. These results were compared with cells exposed to equivalent amounts of growth factor diluted from frozen stocks.

Statistics—All data are expressed as the mean ± S.E. of at least triplicate wells or vials. Statistical comparisons were performed using Student's *t* test or analysis of variance where appropriate. Data were statistically significantly different when p values were <0.05.

RESULTS

Release Kinetics—Controlled release devices were constructed to release TGF-β1 and bFGF with similar kinetics. Two different devices were used because TGF-β1 is not significantly heparin-binding (42), and bFGF is denatured by the methylene chloride (18, 22) required to solvent-cast EVAc release devices (40). Virtually all (>94.1%) of the bFGF was denatured by the organic solvents used to fabricate EVAc matrix devices (18, 22), while TGF-β1 retained its biologic activity when exposed to methylene chloride fumes. When compared with controls, there was no statistically significant difference in the inhibition of endothelial cells (78.1%, $p < 0.0001$) by TGF-β1 or methylene chloride-treated TGF-β1 (85.0%, $p < 0.0001$).

bFGF was released with first order kinetics from heparin-Sepharose beads embedded within calcium alginate microspheres. Repeated binding of bFGF to immobilized heparin encapsulated in the microspheres provided for sustained kinetics (Fig. 1). TGF-β1 did not bind significantly to the heparin, and its release from these beads was too rapid to be of interest. When TGF-β1 was solvent-cast within EVAc microspheres, first order release kinetics were observed, which were consistent with previous studies (40). Approximately 12% of the compound released within the first 8 days (Fig. 1). Alginate/heparin-Sepharose microspheres without bFGF incorporation and EVAc-BSA-TGF-β1 microspheres without TGF-β1 incorporation had no effect on cell growth (p = not significant) (data not shown).

bFGF—Alginate/heparin-Sepharose microspheres with and without bFGF were placed in wells containing sparsely plated (4×10^3 cells/ml/well) endothelial and smooth muscle cells. The controlled release of bFGF significantly increased endothelial cell proliferation beyond that observed with bolus infusion (Fig. 2*A*). While the bolus increased cell number 1.8-fold from control values of $3.4 \pm 0.12 \times 10^4$ cells/ml to $6.0 \pm 0.23 \times 10^4$ cells/ml

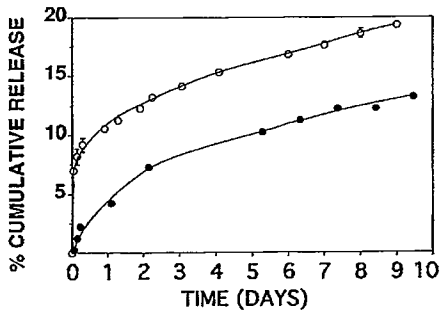

FIG. 1. **Controlled release of bFGF from alginate/heparin-Sepharose microspheres and of TGF-β1 from EVAc microspheres.** Shown is the percent cumulative release of ^{125}I-bFGF (○) from alginate/heparin-Sepharose microspheres and of ^{125}I-TGF-β1 (●) from EVAc microspheres during a 9-day time period. Physical release of bFGF was determined by measuring the presence of ^{125}I-bFGF in the release buffer of three alginate/heparin-Sepharose microspheres as described by Nugent *et al.* (18). Physical release of TGF-β1 was determined by measuring the presence of ^{125}I-TGF-β1 in the release buffer of 5–10 EVAc-BSA-^{125}I-TGF-β1 microspheres. Each data point represents the mean ± S.E. of three identical vials.

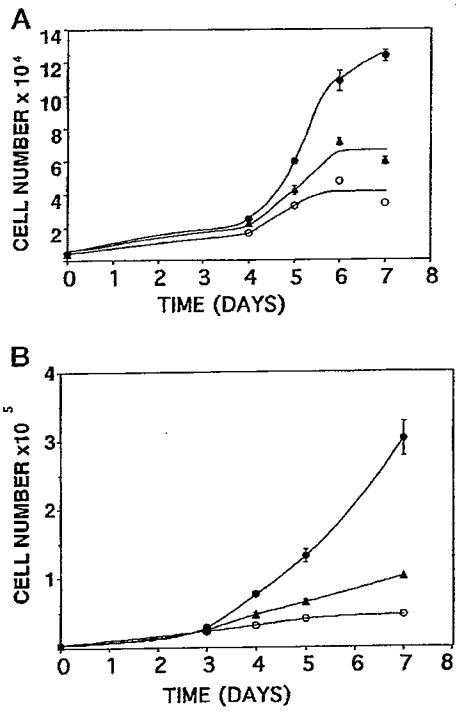

FIG. 2. **Controlled release of bFGF provides optimal endothelial and smooth muscle cell proliferation.** Endothelial (*A*) and smooth muscle (*B*) cells were plated at 4×10^3 cells/ml/well and grown to near confluency in medium alone (○) or were exposed to 8.9 ng of bFGF administered as a single bolus (▲) or released in a sustained fashion from alginate/heparin-Sepharose microspheres (●) over 7 days. Each data point represents the mean ± S.E. of four identical wells. Differences between control, microsphere, and bolus groups are all significant for both cell types ($p < 0.0001$).

($p < 0.0001$), microspherical controlled release increased cell number 3.5-fold to $1.2 \pm 0.04 \times 10^5$ cells/ml ($p < 0.0001$). Smooth muscle cells demonstrated an even greater proliferative response to the sustained release of bFGF in comparison with an equivalent bolus infusion (Fig. 2*B*). The controlled release of bFGF increased cell number 6.5-fold over control

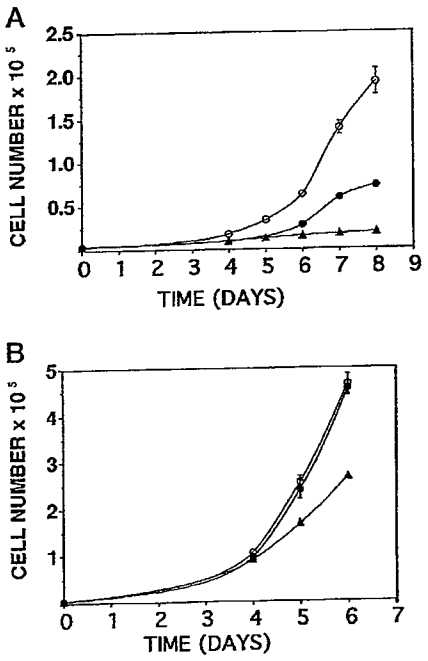

FIG. 3. **Bolus administration of TGF-β1 produces maximal inhibition of endothelial and smooth muscle cell proliferation.** Endothelial (*A*) and smooth muscle (*B*) cells were plated at 4×10^3 cells/ml/well and grown to near confluency in medium alone (○) or in the presence of 0.4 ng of TGF-β1 administered as a single bolus (▲) or sustain-released from one EVAc-BSA-TGF-β1 microsphere (●). Each data point represents the mean ± S.E. of three identical wells. Differences between control, microsphere, and bolus groups are all significant ($p < 0.0001$), except for smooth muscle cell control and microsphere values (p = not significant).

values ($3.0 \pm 0.25 \times 10^5$ cells/ml *versus* $4.6 \pm 0.14 \times 10^4$ cells/ml; $p < 0.0001$), while bolus stimulation produced only a 2.2-fold increase ($1.0 \pm 0.01 \times 10^5$ cells/ml; $p < 0.0070$).

As we (18, 22) and others (43) have previously demonstrated, polymeric device encapsulation can reduce growth factor biologic activity if an inappropriate choice of materials and formulations is used. When bFGF was embedded within and released from the polymeric devices used, the growth factor remained as biologically effective as equivalent compounds diluted from identical stock concentrations throughout the time course of the release experiments. The 0.3 ng of bFGF released from the alginate/heparin-Sepharose microspheres between 0.5 and 1 h produced a 2.9-fold increase in cell number over control values ($2.3 \pm 0.10 \times 10^5$ cells/ml *versus* $7.9 \pm 0.17 \times 10^4$ cells/ml; $p < 0.0001$), while the same amount of bFGF released between days 5 and 6 demonstrated a 3.0-fold increase in cell number over control cells ($2.1 \pm 0.18 \times 10^5$ cells/ml *versus* $7.1 \pm 0.34 \times 10^4$ cells/ml; $p < 0.0001$).

TGF-β1—TGF-β1 is a potent inhibitor of endothelial cell proliferation (Fig. 3*A*). A bolus dose as little as 0.4 ng/ml/well demonstrated a 10.0-fold inhibition of cell proliferation. Control endothelial cells grew 47.5-fold ($1.9 \pm 0.15 \times 10^5$ cells/ml) in 8 days from plating densities of 4×10^3 cells/ml/well ($p < 0.0001$). A 0.4-ng bolus of TGF-β1 restrained cell growth to only a 4.75-fold increase ($1.9 \pm 0.03 \times 10^4$ cells/ml; $p < 0.0001$). Control-released TGF-β1 was a 4-fold less stimulus to endothelial cells than bolus administration. One EVAc-BSA-TGF-β1 microsphere releasing 0.4 ng of TGF-β1 demonstrated only a 2.6-fold inhibition of cell proliferation ($p < 0.0001$) and allowed for an 18.0-fold increase ($7.2 \pm 0.25 \times 10^4$ cells/ml) in cell

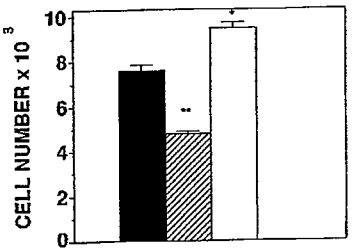

FIG. 4. **Effects of sequential TGF-β1 bolus administration on smooth muscle cell proliferation.** Smooth muscle cells were plated at 4×10^3 cells/ml/well and grown for 6 days in medium alone (■) or in the presence of 2.5 ng of TGF-β1 administered as a single bolus (▨) or exposed to five consecutive daily 0.5-ng bolus amounts of TGF-β1 (□). Each data *bar* represents the mean ± S.E. of four identical wells. Differences between control and single (**, $p < 0.0001$) or consecutive (*, $p < 0.0014$) bolus amounts are all significant.

number over the original plating density ($p < 0.0001$). Smooth muscle cells were not as strongly inhibited by TGF-β1 as were endothelial cells, but exhibited an even greater differential effect with mode of delivery (Fig. 3*B*). A 0.4 ng/ml/well bolus demonstrated a 1.8-fold inhibition of smooth muscle cell proliferation. While control smooth muscle cells with no TGF-β1 increased 115.0-fold ($4.6 \pm 0.2 \times 10^5$ cells/ml) above original plating densities of 4×10^3 cells/ml ($p < 0.0001$), the bolus allowed for cell numbers to increase only 65.0-fold ($2.6 \pm 0.02 \times 10^5$ cells/ml; $p < 0.0001$). The controlled release of TGF-β1 from an EVAc-BSA-TGF-β1 microsphere did not demonstrate a statistically significant effect on cell growth. Cell number was not changed from control values ($4.5 \pm 0.15 \times 10^5$ cells/ml).

Released TGF-β1 also retained its biologic activity throughout the experiment. The 0.03 ng of TGF-β1 released after the initiation of the experiment at 1 h produced an 11.2-fold decrease in cell number over control values ($2.5 \pm 0.29 \times 10^4$ cells/ml *versus* $2.8 \pm 0.09 \times 10^5$ cells/ml; $p < 0.0001$). The same amount of TGF-β1 released at the end of the experimental kinetics profile demonstrated a statistically indistinguishable 10.8-fold decrease in cell number when compared with control values ($4.0 \pm 0.22 \times 10^4$ cells/ml *versus* $4.3 \pm 0.47 \times 10^5$ cells/ml; $p < 0.0001$).

Sequential TGF-β1 Bolus Administration—To determine if the inhibitory effects of a large bolus administration of TGF-β1 could be achieved by the additive effects of smaller bolus additions, smooth muscle cells were exposed to either a 2.5-ng bolus of TGF-β1 or 0.5-ng TGF-β1 bolus additions for 5 consecutive days. Cell number was assessed the following day (Fig. 4). On day 6, smooth muscle cells exposed to the single large bolus already showed a 1.6-fold inhibition of cell proliferation compared with control cells ($4.8 \pm 0.1 \times 10^3$ cells/ml *versus* $7.6 \pm 0.3 \times 10^3$ cells/ml; $p < 0.0001$). Cells exposed to consecutive small bolus additions were not inhibited at all and actually showed a slight 1.3-fold increase in cell number over control values ($9.5 \pm 0.2 \times 10^3$ cells/ml; $p < 0.0014$). An approximately equal, single bolus amount of TGF-β1 (0.4 ng) inhibited smooth muscle cell proliferation 1.8-fold (Fig. 3*B*), but if this approximate bolus amount (0.5 ng) was administered in a controlled release fashion, the inhibitory effect of TGF-β1 was not observed (Fig. 4).

bFGF and TGF-β1 Release from the Extracellular Matrix—^{125}I-bFGF was incorporated into the extracellular matrix and then released over a 10-day time period (Fig. 5). Smooth muscle cell extracellular matrix was used as a representative matrix, which incorporated 10% of ^{125}I-bFGF. After an initial burst, ^{125}I-bFGF was gradually released in a sustained and predictable fashion at an average rate of 2.8%/day, with a cumulative release of 63% at day 10. ^{125}I-TGF-β1 was incorporated with

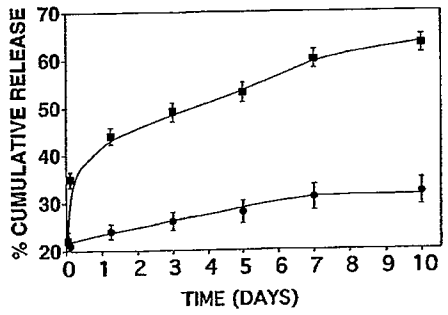

FIG. 5. **Controlled release of bFGF and TGF-β1 from the extracellular matrix.** Shown is the percent cumulative release of ^{125}I-bFGF (■) or ^{125}I-TGF-β1 (●) during a 10-day time period. Physical release of the growth factor was determined by measuring the presence of ^{125}I-bFGF or ^{125}I-TGF-β1 in the release buffer from smooth muscle cell extracellular matrix incorporated with ^{125}I-bFGF or ^{125}I-TGF-β1. Each data point represents the mean ± S.E. of three identical wells.

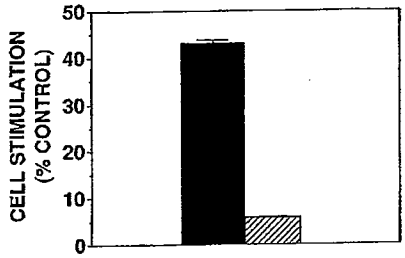

FIG. 6. **Cellular response to bFGF released from the extracellular matrix.** bFGF was incorporated into smooth muscle cell extracellular matrix and released into buffer that was collected every hour and replaced with fresh buffer. The aliquots collected during the second hour of release (■) were placed on smooth muscle cells plated at 4×10^3 cells/ml/well along with equivalent bolus amounts from frozen stock (▨). Cells were counted on day 7. Each data *bar* represents the mean ± S.E. of three identical wells. Differences between extracellular matrix release and bolus groups are significant ($p < 0.0001$).

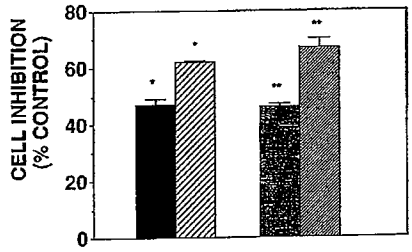

FIG. 7. **Cellular response to TGF-β1 released from the extracellular matrix.** TGF-β1 was incorporated into smooth muscle cell extracellular matrix and released into buffer that was collected every hour and replaced with fresh buffer. The aliquots collected during the second hour of release (■, ▨) were placed on endothelial (■,) and smooth muscle (▩, ▨) cells plated at 4×10^3 cells/ml/well along with equivalent bolus amounts from frozen stock (, ▨). Cells were counted on day 8. Each data *bar* represents the mean ± S.E. of three identical wells. Differences between extracellular matrix release and bolus groups for both cell types are significant (*, $p < 0.0014$; **, $p < 0.0001$).

far higher efficiency (38%), but remained bound to the matrix. Only 1.1% of the growth factor was released per day (Fig. 5). Similar results for ^{125}I-TGF-β1 were observed using endothelial cell extracellular matrix. The matrix incorporation of radiolabeled growth factors is representative of nonradiolabeled growth factor matrix incorporation.

Growth factor interaction with the extracellular matrix determined biologic activity. bFGF released from an extracellular matrix from the first to second hour of release after incorporation demonstrated a 43% increase in smooth muscle cell proliferation compared with control values ($p < 0.0001$) (Fig. 6). An equivalent bolus amount of growth factor diluted from frozen stock produced a 6% increase in cell proliferation compared with control values ($p < 0.0001$). The amount of bFGF released from the extracellular matrix was below the minimal dosage required to elicit an endothelial cell response.

The biologic activity of TGF-β1 was retained after interaction with the extracellular matrix, but the ability of matrix-released TGF-β1 to elicit its biologic effect decreased in contrast to matrix-released bFGF. Endothelial and smooth muscle cell proliferation was inhibited 62 and 67%, respectively, by bolus amounts of TGF-β1, but was inhibited only 47 and 46%, respectively, by extracellular matrix-released TGF-β1 (Fig. 7). Bolus and controlled release groups were statistically different from each other for both cell types (*, endothelial cells, $p < 0.003$; **, smooth muscle cells, $p < 0.0001$).

To preclude the possibility that the extracellular matrix itself would contribute to stimulatory or inhibitory effects by releasing its own endogenous growth factors, endothelial and smooth muscle cells were exposed to release buffer from smooth muscle cell extracellular matrix without the addition of exogenous bFGF or TGF-β1. Cell growth was not statistically different from cells that were grown in medium alone (data not shown).

DISCUSSION

The binding and release of growth factors from the extracellular matrix have been proposed as a means of protecting, sequestering, and optimizing the biologic effect of these compounds (7, 10–15). It is now accepted that cell regulation requires controlled and sustained release; consequently, great efforts have been directed at ensuring prolonged kinetics of administration (21, 22, 44). In fact, growth factors have become the favorite compound with which to evaluate the efficacy of polymeric controlled drug delivery systems (18, 22, 23). To date, however, few have verified and virtually none have questioned the underlying assumption that sustained presentation is the optimum means by which to regulate cell growth. We now demonstrate that for some growth factors sustained delivery is a far less desirable mode of delivery than bolus administration. While the sustained release of bFGF was up to 3.0-fold more potent at increasing vascular endothelial and smooth muscle cell proliferation than bolus administration, the reverse was true for TGF-β1. A bolus of TGF-β1 inhibited vascular cells up to 3.8-fold more efficiently than the same amount of TGF-β1 if control-released. Both growth factors bound to the extracellular matrix, but only bFGF was released in a controlled fashion (2.8%/day). TGF-β1 was absorbed within the matrix and remained tightly adherent. Contact with the extracellular matrix and subsequent release enhanced bFGF mitogenicity, but diminished TGF-β1 activity. Thus, it appears that sustained growth factor release may be the preferable mode of administration only when a similar mode of metabolism is utilized endogenously. Reversible binding to the matrix may indeed signal an endogenous form of controlled release, but this phenomenon must be distinguished from irreversible absorption.

Model of Events—bFGF is exceedingly sensitive to denaturation (7, 12), lacks a signal sequence, and must interact with its receptor for up to 12 h to have its full biologic effect (45, 46). Some alternative mechanism must therefore exist for the presentation of this growth factor to the cell, and matrix binding and release have been suggested as fulfilling this role. bFGF binds avidly to heparan sulfate proteoglycans (5–9, 11, 21, 47). These complexes, which are richly abundant within the extra-

cellular matrix, are released by cells. The heparan sulfate proteoglycans protect bFGF from degradation (7, 10, 12), facilitate distribution (4) and kinetics (47), enrich biologic activity (1, 4, 14, 47), and enable its binding to specific tyrosine kinase receptors (15, 48–50). Thus, our observations that bFGF effects are enhanced after exposure to the extracellular matrix and when control-released probably reflect the protection and stabilizing effects of soluble proteoglycans and the depot potential of the matrix proteoglycans. The physicochemical properties and receptor kinetics of bFGF presumably led to the evolution of these natural protective and facilitory mechanisms and support the need for synthetic means of controlled release when consideration is given to their exogenous administration.

In contrast, the reverse is probably true for TGF-β1. Sustained release is a far less optimal means of administering this growth factor than bolus injection, and its matrix binding likely reflects a very different physiology compared with bFGF. Like bFGF, TGF-β1 has a short half-life and is prone to denaturation. The charge and hydrophobic properties that make it prone to degradation enable high affinity binding to extracellular matrix components such as type IV collagen, fibronectin, and the proteoglycans (51–55). Yet, TGF-β1 exists in a latent form that is less sensitive to destruction, and it reaches half-maximal receptor binding within 30–60 min and saturation within 4 h (53). Thus, at the outset, one can already imagine that sustained release is far less of an imperative. Matrix binding may therefore have arisen to address a different set of issues. Indeed, it is the latent form of the growth factor that interacts most with the extracellular matrix. Dimers of TGF-β1 capped by TGF-β1 latency-associated protein can bind to the specific latent TGF-β1-binding protein through disulfide linkage. Latent TGF-β1-binding protein is rapidly secreted from cells, and the major fraction is covalently associated with the extracellular matrix. The 3-fold complex structure of latent TGF-β1, TGF-β1 latency-associated protein, and latent TGF-β1-binding protein can then bind to the matrix, where it has been postulated that it serves to target the growth factor to the extracellular matrix and create pools of latent TGF-β1 that act as negative feedback regulators. Indeed, we now show that unlike bFGF, extracellular matrix-bound TGF-β1 is not released to any discernible extent, even though binding to the TGF-β1 receptor is reversible (53). Proteolytic cleavage can release all of the three components of the TGF-β1 complex, yet like TGF-β1 interaction with heparin (42), interaction with the matrix does not augment biologic effect.

Thus, both bFGF and TGF-β1 bind to the extracellular matrix, but while the former is released in an augmented form in a prolonged manner, the latter is bound unless cleaved, and its association may even diminish its effects. Endogenous matrix binding may therefore protect, sequester, and then release bFGF, but also permanently bind TGF-β1 to target this growth factor to the matrix and act as a form of feedback regulation. Cells also recognize these two growth factors differently. Vascular endothelial and smooth muscle cells respond optimally to the continuous presentation of the growth factor that is naturally continuously released and to bolus administration of the growth factor that is irreversibly matrix-bound.

Controlled Release of Growth Factors—These data have profound ramifications for the huge industry that has been spawned for the controlled release of growth factors (23). Growth factors were the first compounds used to demonstrate the efficacy of polymer-based controlled release devices. Innovations in polymer chemistry and materials science technology that enabled the design and formulation of controlled release devices occurred at the same time that growth factors were being identified, characterized, purified, and cloned. Moreover, growth factors were ideal compounds to demonstrate the biologic effect of release devices because they are rapidly denatured, active in minute quantities, often difficult to isolate, and of great potential clinical and scientific benefit. Controlled delivery of epidermal growth factor (56, 57) has been reported to stimulate fibroblast proliferation (58), wound healing (59, 60), angiogenesis (61), and neovascularization (62). Recombinant platelet-derived growth factor has been released from collagen discs to promote tissue generation (63). Sustained release of nerve growth factor has been shown to stimulate neurite sprouting from PC12 cells (64), to prevent choline acetyltransferase loss (65, 66), and to prolong survival of adrenal medullary and other brain tissue (67, 68). The effects of control-released insulin on fibroblast proliferation have also been reported (58). TGF-β1 (69–71) and bone-derived growth factors that contain TGF-β1 (72, 73) have been utilized in controlled release systems in models of bone and wound healing (59, 74–77), angiogenesis (78), regulation of ocular growth (79), regulation of mammary gland (43) and epithelial cell (80) growth, and inducible expression from intracardiac grafts to the heart (81). The controlled release of bFGF has been shown to be effective in cell culture (18, 20, 82) as well as angiogenic in corneal tissue (61, 83), retinal regeneration (84), and peripheral nerve regeneration (85). Yet, few of these studies compared the effects of sustained release formulations and bolus delivery.

Various polymeric devices have been used for the controlled release of a number of growth factors. A commonly used system for growth factor release is ethylene-vinyl acetate copolymer (40, 43, 61, 62, 64, 65, 67, 84–87), although the organic solvents used in the preparation of this device destroy the biologic activity of molecules such as bFGF (18, 22). TGF-β1 has been delivered for the purpose of bone repair via the biodegradable polymer poly-(DL-lactide-co-glycolide) and demineralized bone matrix (69, 71, 70, 73), although problems of immunocompatibility, osteoinductivity, and osteoconductivity exist. Other polymer materials have included poly(methyl methacrylate) (58), Pluronic F-127 poloxamer gel (74), polyethylene glycol (88), collagen (63, 77, 89), methylcellulose (75), chitosan (19, 82), cyclodextrin (20), lipids (90), Hydron (78, 83, 91), and other hydrogels, but these all have various limitations such as shorter or suboptimal release times and difficulty of handling. Microspheres consisting of the biocompatible and biodegradable polymer alginate have also been utilized for controlled release delivery of some growth factors. Alginate-polylysine-alginate microspheres were used for the delivery of nerve growth factor (66), and sodium alginate microspheres for the delivery of endothelial cell growth factor (57) and bFGF (18, 22, 86). TGF-β1 release from sodium alginate microspheres has been demonstrated as a potential oral gastrointestinal drug delivery system, in which TGF-β1 is completely and rapidly released within 2 h after a low pH environment is changed to pH 7.4 (80). Our data now support the notion that in considering the burgeoning technology of controlled release, the different interactions these growth factors have with the extracellular matrix must be taken into account. Sustained release should be reserved for those growth factors that are naturally sustain-released.

Conclusion—These data illustrate that the relative functional potencies of growth factors are not dictated by molecular interactions alone, but by other parameters such as the absolute number of receptors, the ability of receptor down-regulation (92), growth factor availability (93, 94), and requirements for receptor activation. Growth factors may bind reversibly or absorb irreversibly to the extracellular matrix in response to very different needs and with very different consequences. This adds flexibility to the cell, enabling it to control the concentra-

tion of drug at its surface by regulating the kinetics of drug presentation and possibly surface receptor expression in concert with the more standard resolution achieved through local dose and binding affinity. Continued elucidation of these interactions will enhance our understanding of growth factor physiology (95, 96), *e.g.* to include the cellular processing of the released growth factors by living cells and tissue. Only by examination of the natural means by which endogenous mediators of growth are metabolized can we fully understand growth factor biology, derive insight into the pathophysiology of proliferative diseases, and provide rational means for their administration and appropriate rules for construction of devices for their controlled and sustained release if indicated.

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[54] METHODS AND COMPOSITIONS OF GROWTH CONTROL FOR CELLS ENCAPSULATED WITHIN BIOARTIFICIAL ORGANS

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[51] **Int. Cl.**[6] **C12N 5/00**

[52] **U.S. Cl.** **435/325**; 435/375; 435/377; 435/400

[58] **Field of Search** 435/240.2, 240.22, 435/240.23, 240.242, 240.243, 325, 375, 377, 400

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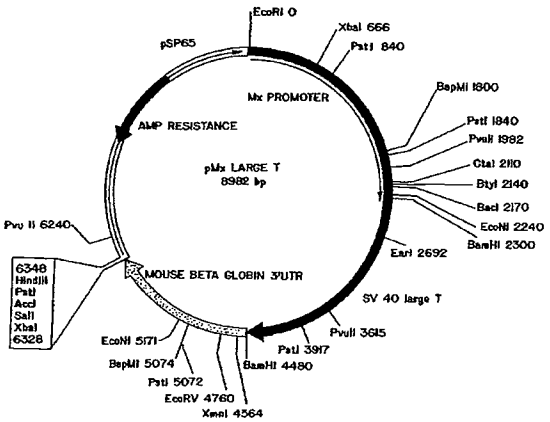
[57] **ABSTRACT**

This invention relates to methods and compositions of controlling cell distribution within a bioartificial organ by exposing the cells to a treatment that inhibits cell proliferation, promotes cell differentiation, or affects cell attachment to a growth surface within the bioartificial organ. Such treatments include (1) genetically manipulating cells, (2) exposing the cells to a proliferation-inhibiting compound or a differentiation-inducing compound or removing the cells from exposure to a proliferation-stimulating compound or a differentiation-inhibiting compound; exposing the cells to irradiation, and (3) modifying a growth surface of the BAO with ECM molecules, molecules affecting cell proliferation or adhesion, or an inert scaffold, or a combination thereof. These treatments may be used in combination.

7 Claims, 5 Drawing Sheets



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Yi, P.N., et al., "Relationship Between Mitotic Delay and the Minimum Dose Rate of X Irradiation Required To Stop Cell Proliferation," *Radiation Research*, 133, pp. 163–169 (1993).

Farghali et al., The concept of Application of Immobilized and Perfused Mammalian cells (a bioreactor model) in biomedical research, *Physiological Research*, 43, pp. 117–120 (1994).

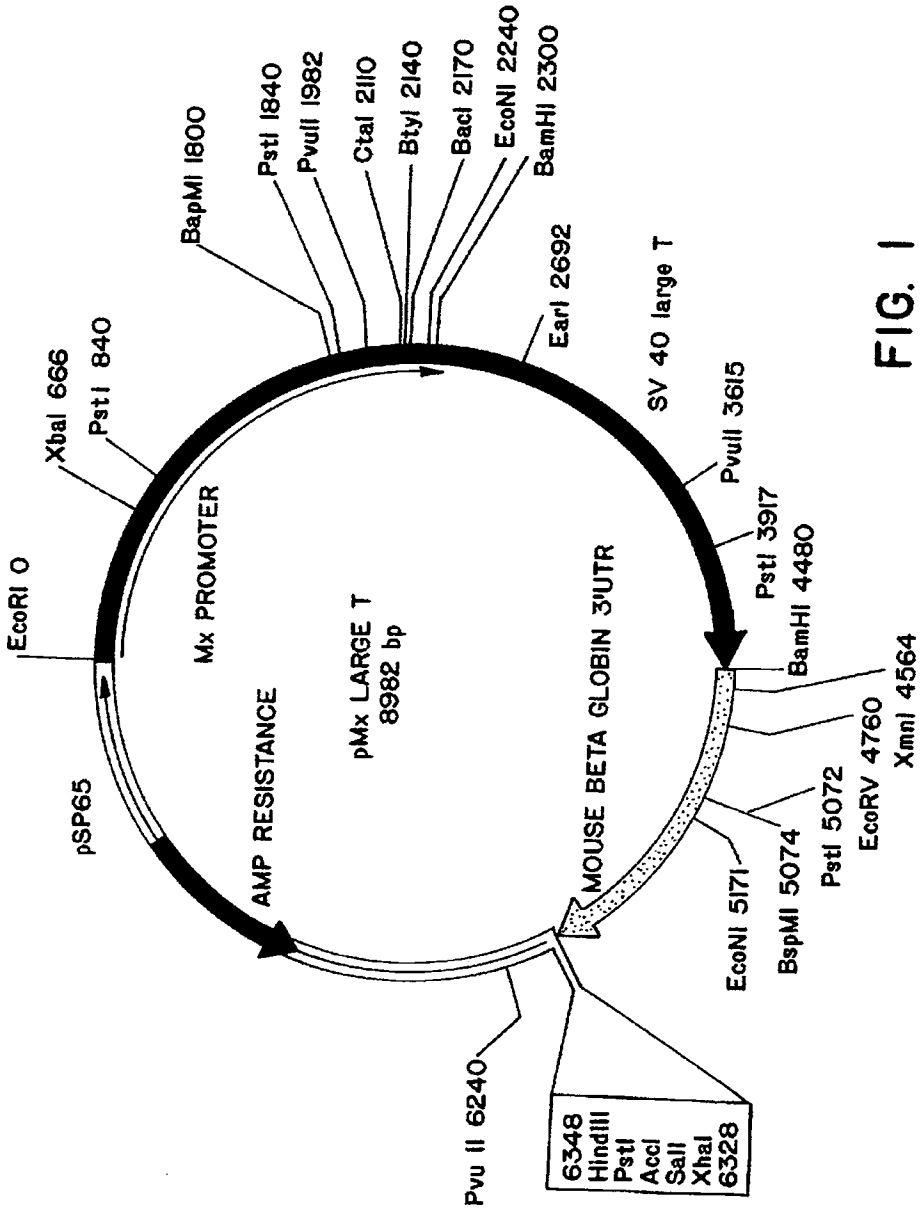
BapMI 1800
PstI 1840
PvuII 1982
CtaI 2110
BtyI 2140
BacI 2170
EcoNI 2240
BamHI 2300
EarI 2692
SV 40 large T
PvuII 3615
PstI 3917
BamHI 4480
XmnI 4564
EcoRV 4760
PstI 5072
BspMI 5074
EcoNI 5171
MOUSE BETA GLOBIN 3'UTR
pMx LARGE T
8982 bp
Mx PROMOTER
AMP RESISTANCE
pSP65
EcoRI 0
XbaI 666
PstI 840
Pvu II 6240
6348
HindIII
PstI
AccI
SalI
XhaI
6328

FIG. 1

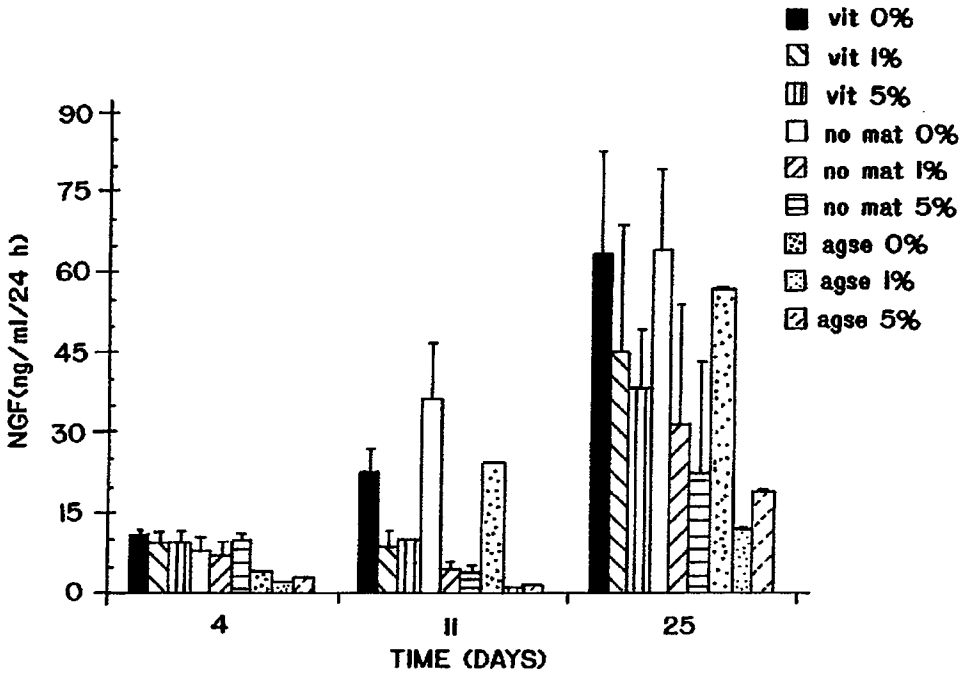
vit 0%
vit 1%
vit 5%
no mat 0%
no mat 1%
no mat 5%
agse 0%
agse 1%
agse 5%
NGF(ng/ml/24 h)
90
75
60
45
30
15
0
4
11
25
TIME (DAYS)

FIG. 2

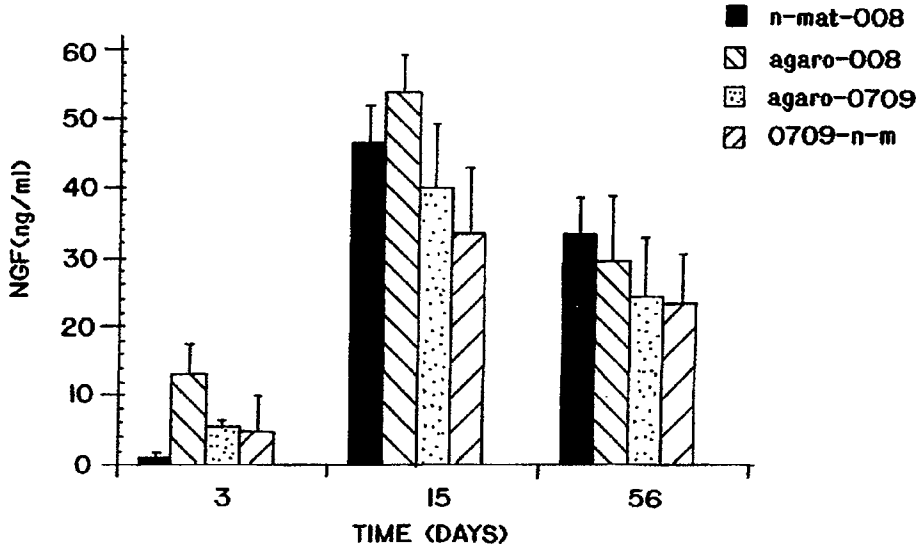
n-mat-008
agaro-008
agaro-0709
0709-n-m
NGF(ng/ml)
60
50
40
30
20
10
0
3
15
56
TIME (DAYS)

FIG. 3

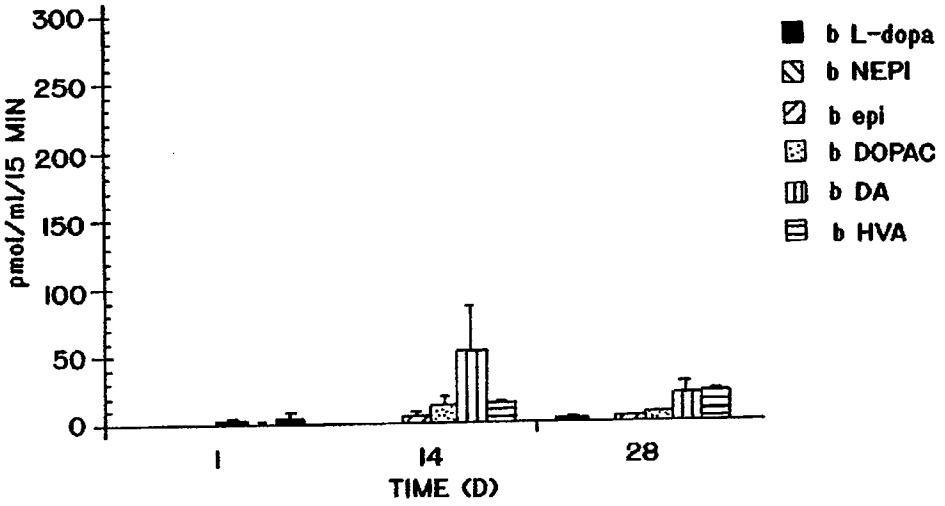
300
250
200
150
100
50
0
pmol/ml/15 MIN
1
14
28
TIME (D)
b L-dopa
b NEPI
b epi
b DOPAC
b DA
b HVA

FIG. 4A

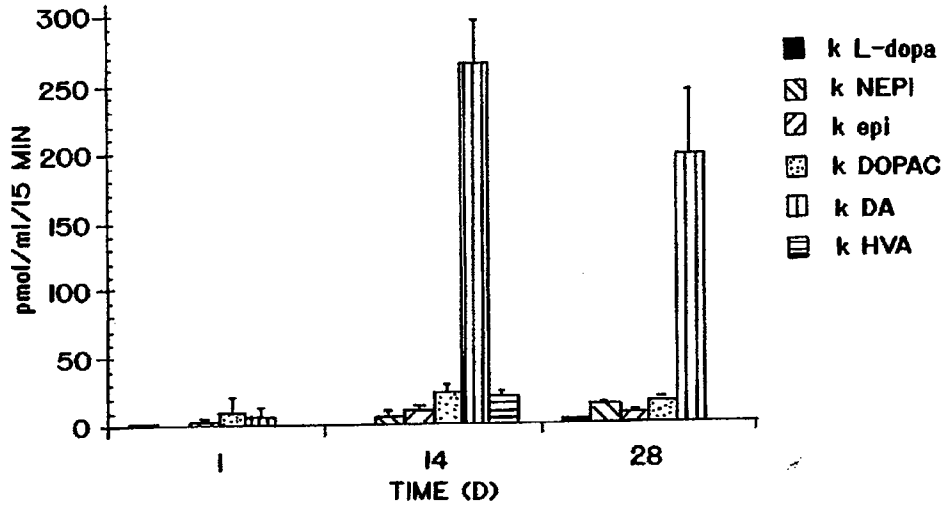
300
250
200
150
100
50
0
pmol/ml/15 MIN
1
14
28
TIME (D)
k L-dopa
k NEPI
k epi
k DOPAC
k DA
k HVA

FIG. 4B

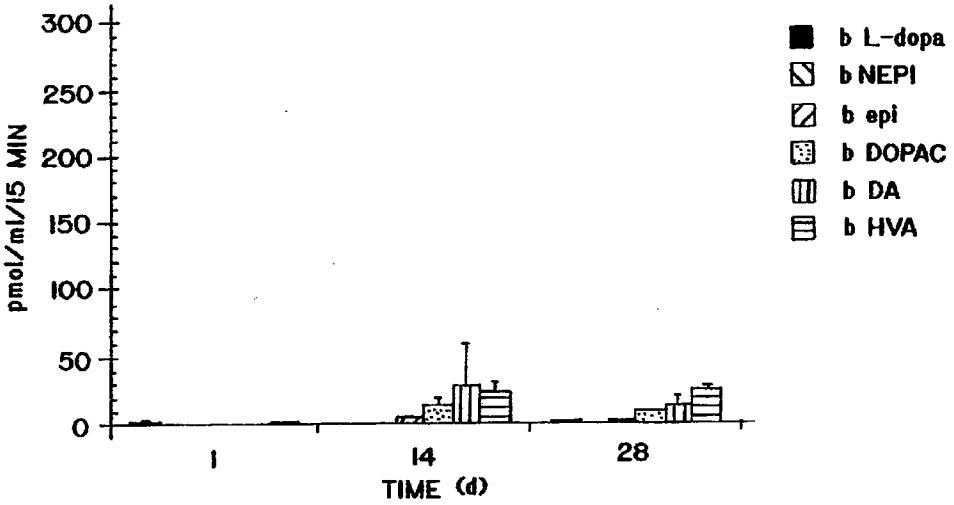
300
250
200
150
100
50
0
pmol/ml/15 MIN
1
14
28
TIME (d)
b L-dopa
b NEPI
b epi
b DOPAC
b DA
b HVA

FIG. 5A

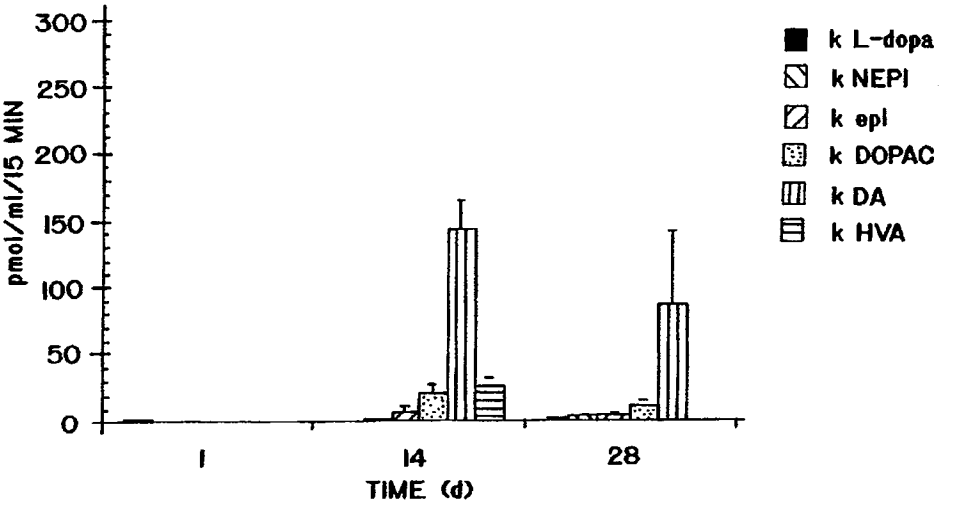
300
250
200
150
100
50
0
pmol/ml/15 MIN
1
14
28
TIME (d)
k L-dopa
k NEPI
k epi
k DOPAC
k DA
k HVA

FIG. 5B

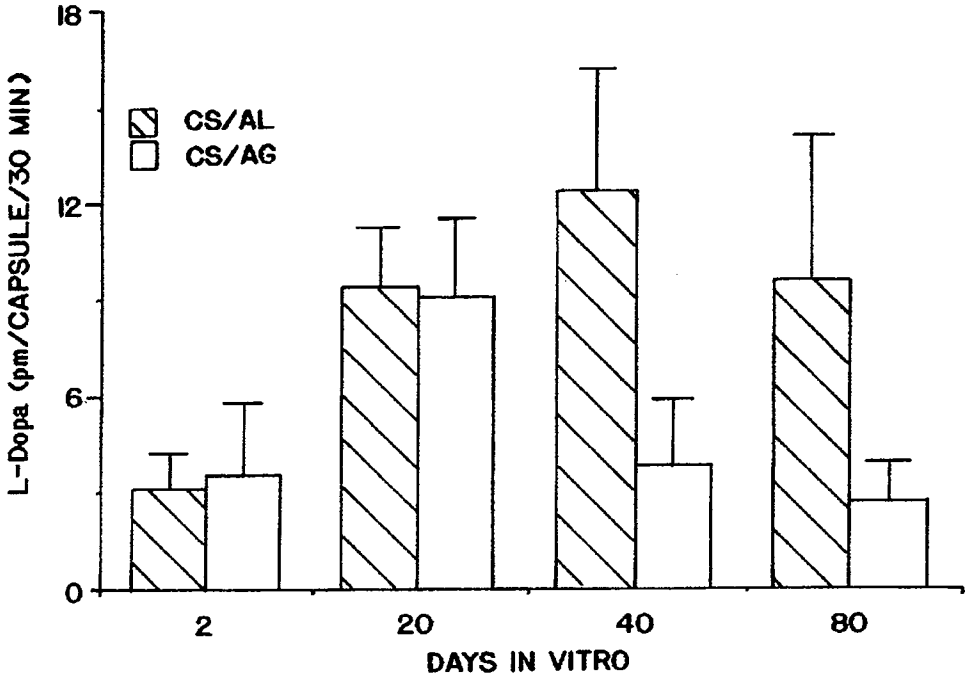
18
12
6
0
L-Dopa (pm/CAPSULE/30 MIN)
CS/AL
CS/AG
2
20
40
80
DAYS IN VITRO

FIG. 6

METHODS AND COMPOSITIONS OF GROWTH CONTROL FOR CELLS ENCAPSULATED WITHIN BIOARTIFICIAL ORGANS

FIELD OF THE INVENTION

This invention relates to methods and compositions for controlling growth of cells encapsulated in a bioartificial organ.

BACKGROUND OF THE INVENTION

Bioartificial organs "BAO" are devices which contain living cells and are designed to provide a needed metabolic function to a host.

The cells encapsulated in BAOs supply one or more biologically active molecules to the host that may be used to prevent or treat many clinical conditions, deficiencies, and disease states.

For example, BAOs containing insulin secreting cells may be used to treat diabetes. Similarly other diseases such as hypoparathyroidism and anemia may be treated by using cells which secrete parathyroid hormone and erythropoietin, respectively.

Bioartificial organs may also be used to supply biologically active molecules for the treatment or prevention of neurodegenerative conditions such as Huntington's disease, Parkinson's disease, Alzheimer's disease, and Acquired Immune Deficiency Syndrome-related dementia. Additionally, lymphokines and cytokines may also be supplied by BAOs to modulate the host immune system. Other biologically active molecules which may be provided by bioartificial organs include, catecholamines, endorphins, enkephalins, and other opioid or non-opioid peptides that are useful for treating pain. Enzymatic deficiencies may also be treated by using BAOs. Alternatively, the biologically active molecule may remove or eliminate deleterious molecules from the host. For example, a BAO may contain cells which produce a biologically active molecule that can be used to "scavenge" cholesterol from a host.

Various "macrocapsule" BAOs are known. See, e.g., Aebischer (U.S. Pat. No. 5,158,881), Dionne et al. (WO 92/03327), Mandel et al. (WO 91/00119), Aebischer (WO 93/00128). BAOs also include extravascular diffusion chambers, intravascular diffusion chambers, intravascular ultrafiltration chambers, and microcapsules. See, e.g., Lim et al., *Science* 210: 908–910 (1980); Sun, A. M., *Methods in Enzymology* 137: 575–579 (1988); Dunleavy et al. (WO 93/03901) and Chick et al. (U.S. Pat. No. 5,002,661).

Because the cells encapsulated in the BAO provide the needed metabolic function, it is desirable that those cells optimally supply the biologically active molecule that effects that function. Typically, differentiated, non-dividing cells may be preferred over dividing cells for use in BAOs because they allow for the optimal production of the desired biologically active molecule. For example, many differentiated, non-dividing cells produce a greater quantity of a desired therapeutic protein than dividing cells because the expression of differentiation specific genes and cell division are thought to be antagonistic processes. Wollheim, "Establishment and Culture of Insulin-Secreting β Cell Lines," *Methods in Enzymology*, 192, p. 223–235 (1990). Cellular replication capacity decreases as cells differentiate. In many cases, proliferation and differentiation are mutually exclusive. Gonos, "Oncogenes in Cellular Immortalisation and Differentiation," 13, *Anticancer Research*, p. 1117 (1993).

The use of differentiated tissue is advantageous because the functional properties of tissue desired for incorporation into a BAO have most often been defined by the properties of differentiated tissue in vivo. Another advantage to the use of differentiated, non-dividing cells is that the cell number within the BAO will remain relatively constant. This, in turn, leads to more predictable results and stable dosage for the recipient host. Additionally, differentiated cells are better suited for use in BAOs which encapsulate more than one cell type secreting biologically active molecules. In such BAOs, if dividing cells are used, different cell types may grow at different rates, resulting in the overgrowth of one cell type. By using differentiated, non-dividing cells, the relative proportions of two or more synergistic cell types can be more readily controlled.

Although in many instances the use of differentiated cells is advantageous, there have been various problems associated with utilizing differentiated cells directly isolated from mammals.

First, there is the potential contamination of the isolated tissue which may require that the tissue taken from each animal be subjected to costly and time-consuming testing to assure that it is pathogen-free.

Second, tissue can be damaged during isolation due to the use of mechanical or enzymatic isolation procedures in the isolation process. The mechanical manipulations are not always easily standardized, resulting in variability between isolations.

Third, ischemia may occur during isolation causing tissue damage.

Fourth, reproducible yields may be difficult because of variations in tissue donors. For example, the age, sex, health, hormonal status of the source animal can affect the yield and quality of the tissue of interest.

Fifth, sometimes there is not enough source tissue to meet the projected demand for the BAO. This occurs for example, in a case where the source tissue comes from a small sized organ or where the ultimate need for tissue amounts is high. If the source of the isolated tissue is human, there is frequently a severe shortage of donor tissue.

Sixth, in some cases, it is desirable to genetically modify the cells used in the BAO. Non-dividing tissue to date has been difficult to genetically modify in vitro and the yields and properties of the modified cells may be uncertain. Thus, because of the foregoing problems, while the use of differentiated, non-dividing cells is desirable, a need exists for a method of producing and maintaining differentiated, non-dividing cells for encapsulation in BAOs.

Because of these problems, dividing cells and cell lines have been favored for use within BAOs to provide the needed biological function. One important advantage in using dividing cells is that such cells may be grown to large numbers in vitro and screened for pathogens and banked. This allows an almost unlimited supply of tissue for lower production costs. Selection schemes such as cell sorting or cloning may be applied to the cell bank to develop subpopulations with improved characteristics. Additionally, dividing cells and cell lines are more amenable to genetic engineering than differentiated, non-dividing cells. The ability to introduce heterologous recombinant DNA allows many new possibilities for the alteration of the function or phenotype of cells to be encapsulated in the BAO. This in turn provides for a greater diversity of therapeutic uses for BAOs.

However, as discussed supra, the disadvantages in encapsulating continuously dividing cells in a BAO include poor regulation of cell numbers in the device that may result in

less predictability in production of the desired biologically active molecule.

While in most cases it may be desirable to limit or minimize cell growth within the BAO, in other cases, e.g., where the BAO is implanted in a "hostile" environment, it may be desirable to allow the cells to proliferate slowly to maintain cell numbers in the BAO.

There is another problem associated with encapsulating cells in general. A variety of cell types have cell adherent properties such that cells tend to adhere to each other and form dense agglomerations or aggregates, especially if there is no adequate substrate available for the cells. Such cell clusters may develop central necrotic regions due to the relative inaccessibility of nutrients and oxygen to cells embedded in the core, or due to the build up of toxic products within the core. The necrotic tissue may also release excess cellular proteins which unnecessarily flood the host with xeno-proteins or other factors which are detrimental to the surviving cells, e.g., factors which elicit a macrophage or other immune response. This problem may be exacerbated when cells are encapsulated in a BAO with a semipermeable membrane jacket because of diffusional constraints across the membrane. Often less oxygen and fewer host supplied nutrients are available within the BAO. In addition, waste products may accumulate in the BAO.

These dense cellular masses can form slowly into dense colonies of cell growth or form rapidly, upon the reassociation of freshly-dispersed cells or tissue mediated by cell-surface adhesion proteins. Cells or tissues with a high metabolic activity may be particularly susceptible to the effects of oxygen or nutrient deprivation, and die shortly after becoming embedded in the center of a large cell cluster. Many endocrine tissues, which normally are sustained by dense capillary beds, exhibit this behavior; islets of Langerhans appear to be particularly sensitive when encapsulated.

There is a need to have a method and composition for controlling the growth of encapsulated cells which combines the various advantages of both proliferating cells and differentiated, non-dividing cells. The present invention provides methods and compositions whereby cells can be proliferated and expanded indefinitely in vitro and where the balance between proliferation and differentiation can be controlled when the cells are encapsulated within the BAO so that the device performs in the desired manner. This invention thus allows regulation of the cell number within the BAO and may therefore provide improved regulation of the output level of the capsule. This invention also provides methods for controlling the growth of cells by controlling cell location within the BAO, thereby reducing the formation of undesirable necrotic cell cores in the BAO. Controlling the cell number and cell location within the BAO also provides the advantage of facilitating optimization of the BAO membrane and other device paramaters to the particular encapsulated cell type. This is because the required device characteristics are more readily determined for a fixed cell population than for a dividing cell population in the BAO. Additionally, long term delivery of biologically active molecules can be achieved.

SUMMARY OF THE INVENTION

The present invention addresses the foregoing problems by providing methods and compositions for controlling the distribution of cells (i.e. cell number or cell location in the BAO, or both) when encapsulated in a BAO. The methods and compositions of this invention include (1) methods and compositions for modification of the cells that are encapsulated within the BAO and (2) methods and compositions for modifying the growth surfaces within the BAO.

Methods and compositions for cellular manipulation include genetic alteration of the cells with a gene which encodes a product that influences cell proliferation or differentiation. The treatment may comprise providing a chemical compound or growth factor which inhibits proliferation or induces differentiation. Alternatively, the treatment may comprise removing from the growth medium a chemical compound or growth factor which stimulates proliferation or inhibits differentiation. The treatment may be before or after encapsulation in the BAO, preferably before encapsulation. Additionally, cell proliferation may be controlled by irradiation.

Methods and compositions for growth surface modification include coating at least one growth surface within the BAO with one or more extracellular matrix molecules ("ECM"). The ECMs may be coated directly onto the luminal surface or any inner support within the BAO, or onto microsphere carriers ("microcarriers"). Cells or cell-seeded microcarriers may additionally be suspended in a matrix material that physically inhibits cell proliferation. Further, the matrix material may be derivatized with chemical or peptide derivatives.

In addition, a growth surface of the BAO can be modified by chemical treatment to inhibit cell attachment or to enhance cell attachment to the BAO's luminal surface. Further, the growth surface can be modified by addition of an inert scaffold prior to cell loading. The scaffold physically inhibits cell outgrowth and provides additional sites for cell attachment. It is to be understood that the various methods and compositions for cell modification and for growth surface modification are not mutually exclusive and may be used in combination.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the plasmid map of a construct containing a 2.3 kb fragment of the murine Mx1 promoter fused to SV40 early region, followed by a BamH1-Xba1 fragment from mouse beta globin 3' untranslated region.

FIG. 2 shows NGF secretion (ng/ml/24 h) after 4, 11 and 25 days from BHK cells encapsulated in control, underivatized membranes (shown as "0%" in legend) or 1% or 5% PEO-PDMS derivatized membranes (shown as "1%" and "5%", respectively, in legend). Cells were encapsulated with no matrix (shown as "no mat" in legend), a Vitrogen™ matrix (shown as "vit" in legend), or an agarose matrix (shown as "agse" in legend).

FIG. 3 shows NGF release from BHK cells grown on CultiSphers™ in the absence of an agarose matrix (legend: n-mat-008, 0709-n-m) or in the presence of an agarose matrix (legend: agaro-008, agaro-0709).

FIG. 4 shows release of catecholamines from PC12A cells at 1, 14 and 28 days after encapsulation in BAOs having a inert PHEMA scaffold. Panel A shows basal catecholamine release; Panel B shows K^+-evoked catecholamine release. The abbreviations L-dopa, NEPI, epi, DOPAC, DA and HVA in the legend represent L-dopa, norepinephrine, epinephrine, dopac, dopamine, and homovanillic acid, respectively.

FIG. 5 shows release of catecholamines from PC12A cells at 1, 14 and 28 days after encapsulation in BAOs having a inert PHEMA/MMA scaffold. Panel A shows basal catecholamine release; Panel B shows K^+-evoked catecholamine release. The abbreviations L-dopa, NEPI, epi, DOPAC, DA and HVA in the legend represent L-dopa, norepinephrine, epinephrine, dopac, dopamine, and homovanillic acid, respectively.

FIG. 6 shows release of L-dopa from SV40/Dβ4-NGF cells grown on Cultispher™ in the presence of an alginate matrix (legend: CS/AL) or in the presence of an agarose matrix (legend: CS/AG) at 2, 20, 40 and 80 days after encapsulation in BAOs.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, a "bioartificial organ" or "BAO" is a device which may be designed for implantation into a host or which may be made to function extracorporeally and either be permanently or removably attached to a host. A BAO contains cells or living tissues which produce a biologically active molecule that has a therapeutic effect on the host. The BAO, upon implantation in a host recipient, should be biocompatible. Accordingly, the BAO should not elicit a detrimental host response sufficient to render it inoperable or not therapeutically useful. Such inoperability may occur, for example, by formation of a fibrotic structure around the capsule limiting diffusion of nutrients to the cells therein. Detrimental effects may also include rejection of the capsule or release of toxic or pyrogenic compounds (e.g. synthetic polymer by-products) from the BAO to surrounding host tissue.

BAOs comprising encapsulated cells may be constructed with immunoisolatory properties which hinder elements of the host immune system from entering the organ, thereby protecting the cells contained within the bioartificial organ from detrimental immune destruction. The use of a BAO increases the diversity of cell types that can be employed in therapy. In implanted BAOs, the devices, which may or may not be immunoisolatory, usually contain the cells or tissues producing a selected product within a semi-permeable physical barrier which will allow diffusion of nutrients, waste materials, and secreted products into surrounding host tissue and retain the contained cells, but minimize the deleterious effects of the cellular and molecular effectors of immunological rejection. Immunoisolatory properties, however, may not be necessary in all cases (e.g., if the cells are autologous or syngeneic to the host).

A "biologically active molecule" is one which (a) may function within the cell in which it is made or (b) may be expressed on the cell surface and affect the cell's interactions with other cells or biologically active molecules (e.g., a neurotransmitter receptor or cell adhesion molecule), or (c) may be released or secreted from the cell in which it is made and exert its effect on a separate target cell or target molecule in the host (e.g., a neurotransmitter, hormone, growth factor, or cytokine).

As used herein, unless otherwise specified, the term "cells" means cells in any form, including but not limited to cells retained in tissue, cell clusters, and individually isolated cells. The cells used in this invention produce at least one biologically active molecule.

Control of cell distribution within the BAO refers to control of the cell number in the BAO, control of the spatial location of cells within the BAO, or both.

A wide variety of cells may be used in this invention. These include well known, publicly available immortalized cell lines as well as dividing primary cell cultures. Examples of publicly available cell lines suitable for the practice of this invention include, L-6 cells, MDCK cells, LLC-PK cells, β-CH3 cells, C2 cells, by hamster kidney (BHK), Chinese hamster ovary (CHO), mouse fibroblast (L-M), NIH Swiss mouse embryo (NIH/3T3), African green monkey cell lines (including COS-a, COS-1, COS-6, COS-7, BSC-1, BSC-40, BMT-10 and Vero), rat adrenal pheochromocytoma (PC12), rat glial tumor cells (C6), RAJI (human lymphoma) cells, MOPC-31C mouse plasmacytoma cells, MN9D cells, MN9H cells, ripTAg transgenic mouse derived cells, SCT-1, β-TC cells, Hep-G2 cells, AT-T20 cells, beta-cell lines such as NIT cells or RIN cells, Ntera-2 cells (Pleasure et al., *Journ. Neuroscience,* 12, pp. 1802–15 (1992)) and human astrocyte cell lines such as U-373 and U-937.

Primary cells that may be used include, bFGF-responsive neural stem/progenitor cells derived from the CNS of mammals (Richards et al., *PNAS* 89, pp. 8591–8595 (1992); Ray et al., *PNAS* 90, pp. 3602–3606 (1993)), primary fibroblasts, Schwann cells (WO 92/03536), astrocytes, oligodendrocytes and their precursors, myoblasts, and adrenal chromaffin cells.

Cells can also be chosen depending on the particular method of growth control and differentiation to be used. For example, stem cells can easily be used with the methods which induce differentiation by introducing a chemical substance. Generally, stem cells are undifferentiated cells which in vivo are normally quiescent but are capable of proliferation and capable of giving rise to more stem cells having the ability to generate a large number of progenitor cells that can in turn give rise to differentiated or differentiatable daughter cells. Stem cells represent a class of cells which may readily be expanded in culture, and whose progeny may be terminally differentiated by the administration of a specific growth factor. See, e.g., Weiss et al. (PCT/CA 92/00283).

Myoblasts are one type of cell that may be encapsulated in a BAO according to this invention. Myoblasts are muscle precursor cells originally derived from mesodermal stem cell populations. A number of myoblast cell lines are available which can undergo differentiation in culture, e.g., L-6 and β-CH3 cells. Primary myoblasts can be readily isolated from tissue taken from an autopsy or a biopsy, and can be purified and expanded. Myoblasts proliferate and fuse together to form differentiated, multi-nucleated myotubes. Myotubes no longer divide, but continue to produce muscle proteins. While proliferating, myoblasts may readily be genetically engineered to produce therapeutic molecules. Methods are known for introducing one or more genes into myoblasts to produce the desired biologically active molecules. Myoblasts are capable of migrating, fusing into pre-existing fibers, and serving as carriers for the introduced gene(s). Verma et al. (WO 94/01129); Blau, et al., *TIG,* 9, pp. 269–74 (1993); WO 93/03768; WO 90/15863. The engineered cells may then be encapsulated and allowed to differentiate in the BAO.

The choice of cells also depends upon the intended application. The cells within the BAO may be chosen for secretion of a neurotransmitter. Such neurotransmitters include dopamine, gamma aminobutyric acid (GABA), serotonin, acetylcholine, noradrenaline, epinephrine, glutamic acid, and other peptide neuro-transmitters. Cells can also be employed which synthesize and secrete agonists, analogs, derivatives or fragments of neurotransmitters which are active, including, for example, cells which secrete bromocriptine, a dopamine agonist, and cells which secrete L-dopa, a dopamine precursor.

The cells can be chosen for their secretion of hormones, cytokines, growth factors, trophic factors, angiogenesis factors, antibodies, blood coagulation factors, lymphokines, enzymes, and other therapeutic agents or agonists, precursors, active analogs, or active fragments thereof. These include enkephalins, catecholamines, endorphins, dynorphin, insulin, factor VIII, erythropoietin, Substance P, nerve growth factor (NGF), Glial cell line-derived Neu-

rotrophic Factor (GDNF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5, CDF/LIF, bFGF, aFGF, an array of other fibroblast growth factors, ciliary neurotrophic factor (CNTF), and interleukins.

It should be understood from the foregoing that the cells useful in the methods of this invention include untransformed cells that secrete the desired biologically active molecule(s), or cells that can be transformed to do so.

The genes encoding numerous biologically active molecules have been cloned and their nucleotide sequences published. Many of those genes are publicly available from depositories such as the American Type Culture Collection (ATCC) or various commercial sources. Genes encoding the biologically active molecules useful in this invention that are not publicly available may be obtained using standard recombinant DNA methods such as PCR amplification, genomic and cDNA library screening with oligonucleotide probes from any published sequences. Any of the known genes coding for biologically active molecules may be employed in the methods of this invention. See, e.g., U.S. Pat. No. 5,049,493; Gage et al., U.S. Pat. No. 5,082,670; and U.S. Pat. No. 5,167,762.

A gene of interest (i.e., a gene that encodes a suitable biologically active molecule) can be inserted into a cloning site of a suitable expression vector by using standard techniques. These techniques are well known to those skilled in the art.

The expression vector containing the gene of interest may then be used to transfect the cell line to be used in the methods of this invention. Standard transfection techniques such as calcium phosphate co-precipitation, DEAE-dextran transfection, lipid-mediated methods, or electroporation may be utilized.

Methods are provided herein to control the growth of dividing cells, whereby the balance between proliferation and differentiation can be controlled to provide a supply of differentiated, non-dividing encapsulated cells within the BAO. Methods are also provided to control the growth of both dividing and non-dividing cells, whereby cell distribution and cell number within the BAO are controlled, resulting in reduced formation of necrotic cell cores and reduced cellular debris.

Control of Proliferation and Differentiation By Genetic Engineering

Methods and compositions are herein provided for controlling cell growth by genetic alteration of cells with a gene encoding a product that influences cell proliferation or differentiation.

According to one aspect of this invention, conditionally immortalized cell lines are used to achieve growth control in the BAO. Primary cells are transformed with a gene encoding a proliferation-promoting product. The proliferation-promoting gene is operatively linked to a regulatable promoter. The techniques described by Land et al., *Nature,* 304, pp. 596–602 (1983) or Cepko, *Neuron,* 1, pp. 345–53 (1988) for producing immortalized cells can be routinely modified to produce conditionally-immortalized cells.

According to this method, cell proliferation (i.e., mitosis) can be inhibited or arrested by decreased expression of a proliferation-promoting gene, such as an oncogene (e.g., c-myc, v-mos, v-Ha-ras, SV40 T-antigen, E1-A from adenoviruses). Reduced expression of the oncogene is achieved by downregulation, repression or inactivation of the promoter driving oncogene expression when the BAO is implanted in vivo in a host. Upregulation, activation or derepression of the regulatable promoter in vitro results in expression of the proliferation-promoting gene, thereby permitting cell proliferation in vitro. Suitable promoters are those which can be downregulated in vivo, including, e.g., glucocorticoid responsive promoters, such as PNMT (Hammang et al., *Neuroprotocols,* 3, pp. 176–83 (1993) and interferon ("IFN")-responsive promoters, such as Mx1 (Hug et al., *Mol. Cell. Biol.,* 8, pp. 3065–79 (1988); Arnheiter et al., *Cell,* 52, pp. 51–61 (1990)), retroviral long terminal repeat promoters, tetracycline responsive promoters, e.g., the lac promoter, and insulin-responsive promoters. See also, McDonnell et al. WO 93/23431. It will be appreciated that choice of promoter will depend upon the intended implantation site. Thus, e.g., glucocorticoid or IFN-responsive promoters are useful for implantation in the brain according to this method, since the levels of glucocorticoid and/or IFN are very low in the brain. Thus, these promoters would not be expected to direct significant levels of expression of the oncogene upon implantation of the BAO in the brain.

In one embodiment, conditionally-immortalized cells are generated by operatively linking an oncogene to a regulatable promoter. The promoter is activated or upregulated in the presence of a binding protein. Production of the binding protein can be regulated by operatively linking the gene encoding the binding protein to a tetracycline responsive promoter.

For example, one embodiment contemplates a transformed cell containing a constitutive promoter driving tet repressor expression. The cell additionally contains a heterologous gene operatively linked to the CMV-IE promoter. If the CMV-IE promoter is flanked with tet operator sequences, expression from this promoter can be turned off by the tet repressor. In the presence of tet, transcription occurs because tet binds with the tet repressor allowing other transcription factors to bind the CMV-IE promoter. According to this embodiment, the oncogene is only expressed when tetracycline is present. Thus, cells can be proliferated in vitro in the presence of tetracycline.

Several days prior to implantation, tetracycline can be removed to reduce transgene expression, and thus correspondingly reduce or halt cell proliferation in the BAO.

In a specific embodiment using conditionally immortalized cells, growth control is achieved using the Mx1 promoter. The Mx1 gene encodes a protein which confers resistance to influenza A and B. The Mx1 gene is tightly regulated by its promoter. In the absence of interferon ("IFN"), the gene is not expressed and the gene is inducible in the presence of IFNα and IFNβ. Arnheiter et al., *Cell,* 52, pp. 51–61 (1990) reported the generation of Mx1 transgenic mice that exhibited interferon inducible expression of the transgene in several tissues. The SV40 large T-antigen is capable of transforming and immortalizing cells derived from a number of tissues.

In one embodiment, the mouse Mx1 promoter can be fused with the SV40 early region and the chimeric gene used to generate transgenic mice. The tight regulation afforded by the Mx1 promoter elements allows one to control oncogene expression in tissues or in cell cultures prepared from the transgenic animals, thereby allowing creation of conditionally-immortalized cell lines.

In the presence of IFNα or IFNβ, the cell lines produced in this manner can be expanded arithmetically as with most other cell lines. Cell division can be halted by removal of IFNα or IFNβ, either before or after encapsulation. In a preferred embodiment, neural stem cells (neurospheres) can be prepared from transgenic mice containing the Mx1-SV40

T-antigen construct using the method of Weiss (PCT/CA 92/00283). The conditionally immortalized neural stem cell line so obtained can then be encapsulated and implanted in vivo in a host.

Additionally, if desired, the conditionally immortalized neural stem cell line can be further genetically modified to release any of a number of growth factors or neurotransmitter molecules, according to standard techniques. Other IFN-responsive promoters may also be useful in this embodiment. These promoters include metallothionein, H-2K^b, H-2D^d, H-2L^d, HLA-A3, HLA-DRα, an HLA class I gene, 202, 56K, 6-16, IP-10, ISG15, ISG54, and 2',5'-oligo (A) synthetase. See, Hug et al., *Mol. Cell. Biol.*, 8, pp. 3065–79 (1988).

This embodiment is particularly suited for cells to be encapsulated in BAOs for implantation in the brain. Circulating levels of IFNα and IFNβ in the brain are sufficiently low that transcriptional activity driven by the Mx1 promoter is insufficient to result in cell proliferation. In the founder transgenic animals, the expression of T-antigen could be induced in several tissues, but the natural expression of the oncogene was seen only in the thymus. However, thymic expression of the oncogene is a relatively common phenomenon in transgenic animals expressing the SV40 early region. Thus, in the absence of significant oncogene expression, the cells can be kept in a near quiescent state in vivo.

Another embodiment makes use of the observation that in traditional retroviral infection techniques to genetically engineer cells for use in vivo, retroviral promoters, e.g., the long terminal repeat ("LTR") promoter, are used. See, e.g., Gage et al. (U.S. Pat. No. 5,082,670). The expression of genes driven by these promoters is typically downregulated in vivo. It is thought that this downregulation is mediated by circulating cytokines. This invention makes use of this normally detrimental downregulation of retroviral genes to stop or decrease cellular proliferation when cells are encapsulated within the BAO and implanted in vivo. In this instance, an immortalizing gene (oncogene) is driven from the LTR. This gene will "immortalize" the cells while they are maintained and expanded in vitro. Following implantation, in the presence of cytokines, the "immortalizing" oncogene is downregulated, proliferation decreases or stops and the cells may become quiescent within the device.

According to this embodiment conditionally-immortalized cells may be produced by retroviral infection or DNA transfection with cDNA containing an oncogene (e.g. c-myc, v-mos, v-Ha-ras, SV40 T-antigen, E1-A from adenoviruses) operatively linked to a retroviral promoter, e.g., the LTR promoter. We prefer Moloney murine leukemia virus (MLV), Rous sarcoma virus (RSV), and mouse mammary tumor virus (MMTV) promoter sequences.

These transformed cells will normally express the oncogene in vitro. Successfully transformed cells will be grown in culture using established culture techniques. LTR-transgene expression can be stimulated by the addition of dexamethasone or epidermal growth factor to shorten the amount of time needed to culture the transformed cells. By exposing the cells to cytokines, e.g., gamma-interferon (IFN-γ), TNF-α and transforming growth factor-β (TGFβ), preferably several days prior to encapsulation and implantation, mitosis can be reduced by hindering LTR-driven transgene expression. Schinstine and Gage, *Molecular and Cellular Approaches to the Treatment of Neurological Disease*, 71, ed. Waxman, S. G. (1993); Seliger et al., *J. Immunol.*, 141, pp. 2138–44 (1988); Seliger et al., *J. Virology*, 61, pp. 2567–72 (1987); Seliger et al., *J. Virology*, 62, pp. 619–21 (1988).

Any suitable cell can be conditionally immortalized according to the above methods. One of ordinary skill in the art can determine the suitability of a given cell type for conditional immmortalization by screening methods well known in the art, including according to the methods provided herein.

Methods are provided herein for growth control of immortalized cell lines or other continuously proliferating cells by transforming these cells to include tumor suppressor genes, e.g., the p53 gene or RB gene, to halt or reduce proliferation. Tumor suppressor genes, or anti-oncogenes, are believed to be growth-constraining genes. See, e.g., Weinberg, *Neuron*, 11, pp. 191–96 (1993). For example, a wild-type p53-activated fragment 1 (WAF1) can suppress tumor cell growth in culture. It is theorized that genes induced by the p53 protein may mediate its biological role as a tumor suppressor. El-Deiry et al., "WAF1, a Potential Mediator of p53 Tumor Suppression," *Cell*, 75, pp. 817–825 (1993). The WAF1 gene is also referred to as the CIP1 gene. Other p53-mediated growth arresting genes include GADD45 and GADD153 (or CHOP). See Ron *Proc. Natl. Acad. Sci. USA*, 91, pp. 1985–86 (1994). The standard techniques for transforming cells with heterologous DNA discussed above can be used here.

According to one embodiment, immortalized cells or continuously proliferating cells are transformed with a tumor suppressor gene operatively linked to a regulatable promoter. Use of a suitable regulatable or inducible promoter allows expression of the transgene to be downregulated or "turned off" when the transformed cells are cultured in vitro, thus permitting expansion. Upon encapsulation and implantation, the promoter is "induced," or upregulated, and expression of the tumor suppressor gene occurs, resulting in reduced or halted cell proliferation.

The tyrosine hydroxylase and erythropoeitin promoters may be useful in this aspect of the invention. These promoters are typically "downregulated" under high O_2 conditions, such as those encountered in vitro, but are "upregulated" under low O_2 conditions, like those that cells encounter upon encapsulation in a BAO and implantation in a host.

In addition, suitable coupled or derepressible promoter systems may be used to achieve the desired regulation of the proliferation-suppressing gene. One suitable system, e.g., involves use of the AP1 promoter and the lac operator/PGK1 promoter system described by Hannan et al., *Gene*, 130, pp. 233–39 (1993). The AP1 promoter is operatively linked to the lac repressor gene. The lacO (lac operator) and 3-phosphoglycerate kinase (PGK1) promoter is operatively linked to the proliferation-suppressing gene. Addition of exogenous phorbol ester in vitro induces the AP1 promoter, resulting in expression of the lac repressor protein. In the presence of repressor protein, the lacO-PGK1 promoter construct is repressed, and no expression of the proliferation-suppressing gene occurs. In the absence of phorbol ester in vivo, no repressor protein is expressed, the lacO-PGK1 promoter is derepressed, and the proliferation-suppressing gene is expressed.

According to one method, a suitable cell is transformed with a gene encoding a differentiation-inducing product. This differentiation-inducing gene is operatively linked to a regulatable promoter. According to this method, the differentiation-inducing gene would be expressed upon encapsulation and in vivo implantation in a host. However, expression can be arrested or inhibited in vitro by appropriate downregulation, repression or inactivation of the regulatable promoter, thus allowing expansion of a desired cell

or cell line in vitro. This method can be used with dividing cells, or primary cells that have been immortalized. High mobility group chromosomal protein 14, "HMG," is one example of a gene involved in regulating differentiation of cells. Any suitable promoter that is upregulated in vivo but which can be "turned off" or downregulated in vitro can be used in this embodiment, as discussed supra for use with proliferation-arresting genes. In addition, any suitable derepressible promoter system can be used, as discussed supra, for the regulation of tumor suppressor gene expression.

Another method of growth control uses antisense RNA or DNA, or their derivatives. Antisense RNA or DNA is a single-stranded nucleic acid which is complementary to the coding strand of a gene or to the "coding" mRNA produced from transcription of that gene. If the antisense RNA is present in the cell at the same time as the mRNA, the antisense RNA hybridizes to the mRNA forming a double strand which then cannot be translated by ribosomes to make protein. Antisense RNA can be administered to cells either via microinjection or bulk addition to culture medium. The preferred method of the instant invention is to transfect target cells with eukaryotic expression vectors. Neckers et al., "Antisense Technology: Biological Utility And Practical Considerations", *Am. J. Physiol.* 265 (*Lung Cell. Mol. Physiol.*, 9), pp. L1–L12 (1993).

According to this embodiment, an antisense gene encoding antisense RNA to either a proliferation-inducing gene or a tumor suppressor gene can be operatively linked to an inducible promoter. When the promoter is induced, antisense RNA is produced. If the transformed cells contain a proliferation-inducing gene, according to this embodiment, antisense RNA production would be halted or downregulated in vitro to allow for cell expansion, and upregulated in vivo, to achieve cessation or reduction of proliferation.

Alternatively, if the transformed cells contain a tumor suppressor gene, antisense RNA production would be upregulated in vitro and downregulated in vivo to achieve the desired growth control.

In addition, antisense technology could be used to construct any antisense gene to a gene encoding a product essential for proliferation or differentiation. Appropriate induction of the expression of the antisense gene would allow one of skill in the art to achieve the desired growth control of encapsulated cells according to this invention.

It is preferred to use a regulatable promoter/gene construct that can be manipulated in vivo in the event that it becomes necessary or desirable to induce further cell proliferation in vivo. For example, in the Mx1/SV40 construct discussed supra, IFN can be added locally or systemically to induce oncogene expression. An increase in cell division in vivo in the BAO may be desirable to increase cell number to replace dead cells in the BAO, or to achieve increased output of the desired biologically active molecule from the BAO.

Control of Growth and Differentiation by Use of Chemical Compounds

According to another method of this invention, cells may be exposed to a treatment which inhibits proliferation or induces differentiation. In some methods, the treatment comprises providing a chemical compound or growth factor. In other methods, the treatment comprises removing a chemical compound or growth factor from the growth medium. The treatment may be before or after encapsulation in the BAO, preferably before encapsulation.

The protein or chemical compound used depends on the cell type and the desired effect. One of ordinary skill in the art could screen a given cell type for its responsiveness to a selected compound or protein, with routine techniques.

In one method, cell distribution is controlled by a treatment that comprises removing a proliferation-inducing chemical compound or growth factor from the cell growth medium. In one embodiment, growth factors, such as epidermal growth factor ("EGF"), transforming growth factor a ("TGF-α"), amphiregulin, or any other suitable agent, can be used to induce proliferation of stem or progenitor cells, including cells from embryonic sympathetic ganglia and immortalized progenitor cells, preferably neural stem cells (Weiss, PCT/CA 92/00283). This allows maintenance and expansion of a supply of neuronal precursor cells in vitro. When encapsulated in the absence of these proliferation-inducing growth factors, the neuronal precursor cells cease dividing and differentiate.

The neuronal precursor cells may be further induced to differentiate by treatment with, e.g., phorbol ester, or growth on a fixed substrate, including ionically charged surfaces such as poly-L-lysine and poly-L-ornithine and the like. Differentiation may also be induced by treatment with a member of the FGF family in combination with at least 1 member of either the ciliary neurotrophic factor (CNTF) or nerve growth factor (NGF) family of factors as described in Ip et al. (WO 94/03199).

In another embodiment, a multilineage growth factor produced in the stroma, also termed "mast cell growth factor," "stem cell factor," "c-kit-ligand," or "Steel factor," can be used to induce proliferation of hematopoietic stem cells. To maintain a supply of dividing cells in vitro, hematopoietic stem cells are cultured in the presence of mast cell growth factor. To arrest or reduce proliferation, the mast cell growth factor is removed from the culture medium. This can be done before or after encapsulation, preferably before encapsulation.

Examples of other multilineage growth factors that promote proliferation include interleukin-3 and granulocyte-macrophage colony-stimulating factor. Mast cell growth factor can also affect cell growth in combination with other multilineage growth factors, or lineage specific growth factors, e.g., erythropoietin. For example, mast cell growth factor is thought to act synergistically with IL-3 in inducing proliferation and differentiation of highly enriched murine hematopoietic stem cells. Galli et al., "The Biology of Stem Cell Factor, a New Hematopoietic Growth Factor Involved in Stem Cell Regulation," *Int. J. Clin. Lab. Res.*, 23, pp. 70–77 (1993).

In another method of this invention, control of cell distribution in the BAO may be achieved by providing a chemical compound or growth factor which inhibits cell proliferation or induces differentiation. Any suitable proliferation-inhibiting or differentiation-inducing compound may be used according to this method.

It will be appreciated that different cell types may respond differently to various chemical compounds. One of ordinary skill in the art can routinely screen a particular compound to determine its effectiveness in affecting proliferation or differentiation of a given cell type.

In one embodiment, cytokines, including, e.g., transforming growth factor β1 (TGFβ1), may be used to arrest or inhibit cell proliferation or to induce cell differentiation. For example, decreased proliferation and enhanced differentiation in BHK cells can be achieved by exposure to TGFβ1 and ascorbate. Similarly, TGFβ1 can be used to induce differentiation in fibroblast cells and also as a growth inhibitor of keratinocytes and endothelial cells. Phillips et al., "Ascorbic Acid and Transforming Growth Factor-β1 Increase Collagen Biosynthesis via Different Mechanisms: Coordinate Regulation of Proα1(I) and Proα1(III)

Collagens," *Archives of Biochemistry and Biophysics,* 295, pp. 397–403 (1992).

In another embodiment, TGFβ1, serotonin, or FGF may be used to control the growth of neuroendocrine cells. The growth of neuroendocrine cells can be regulated by their own products in an autocrine fashion. TGFβ1 is an autocrine growth-inhibitory factor for human pancreatic carcinoid cells (BON), while FGF and serotonin are autocrine growth-stimulatory factors. The inhibitory effect of TGFβ1 on the growth of BON cells can be reversed by addition of serotonin. Townsend Jr. et al., "Studies of Growth Regulation in a Neuroendocrine Cell Line," *Acta Oncologica,* 32, pp. 125–130 (1993).

A variety of other chemicals may also be used according to the methods of this invention to arrest or inhibit proliferation or induce differentiation of cells. These chemicals include mitomycin C, 5-bromo-deoxyuridine (BrdU), prostaglandin E_1 (PGE_1), dibutyryl cAMP, 1-β-D-arabinofuranosyl cytosine (Ara-C), nicotinamide, and heparin. Mitomycin may be particularly suited for controlling proliferation of encapsulated βHC cell lines. See, e.g., Radvanyi et al., *Mol. Cell. Biol.,* 13, pp. 4223–27 (1993).

Sometimes a combination of chemicals can be used. Human neuroblastoma cells IMR-32 may be induced to differentiate in vitro when treated with mitomycin C and BrdU or PGE_1 and dibutyryl cAMP (dbcAMP). Gash et al., "Amitotic Neuroblastoma Cells Used for Neural Implants in Monkeys," *Science,* 233, pp. 1420–22 (1986). Serial pretreatments of human embryonal rhabdomyosarcoma cell line with Ara-C results in marked growth inhibition in vitro, loss of tumorigenicity in vivo, and a more differentiated phenotype even following removal of the compound. Crouch et al., "Ara-C Treatment Leads to Differentiation and Reverses the Transformed Phenotype in a Human Rhabdomyosarcoma Cell Line," *Experimental Cell Research,* 204, pp. 210–16 (1993). Nicotinamide (NIC) is thought to induce differentiation and maturation of human fetal pancreatic islet cells. Otonkoski et al., "Nicotinamide Is a Potent Inducer of Endocrine Differentiation in Cultured Human Fetal Pancreatic Cells," *J. Clin. Invest.,* 92, pp. 1459–66 (1993).

The addition of dbcAMP has also been reported to influence the differentiation of developing tissues. For example, dbcAMP is thought to modulate the differentiation of astrocyte precursors, induce neurite formation in PC12 cells, and stimulate Schwann cell proliferation. Baron-Van Evercooren et al., "Schwann Cell Differentiation in vitro: Extracellular Matrix Deposition and Interaction," *Dev. Neurosci.,* 8, pp. 182–96 (1986). Similarly, differentiation of Schwann cells can be induced by exposure to ascorbate. Ibid.

Further, sialoglycopeptide ("SGP") molecules may be used to inhibit or arrest cell proliferation. For example, an 18 kDa cell surface sialoglycopeptide isolated from intact bovine cerebral cortex cells arrested proliferation of exponentially growing Swiss 3T3 cells. See, e.g., Toole-Simms et al., *Jour. Cell. Physiol.,* 147, pp. 292–97 (1991); Fattaey et al., *Exp. Cell. Res.,* 194, pp. 62–68 (1991). Numerous transformed and untransformed cell types have been shown to be sensitive to some SGPs. These cells include epithelial-like and fibroblast cells from a broad spectrum of vertebrate and invertebrate species. See, e.g., Fattaey et al., *Jour. Cell. Physiol.,* 139, pp. 269–74 (1989) incorporated herein by reference.

It will be appreciated that some of the foregoing treatments may only have a transient effect on proliferation and differentiation. In such cases it may be desireable to provide a continuously replenished supply of the compound or growth factor to the encapsulated cell when implanted in vivo in the host. This can be accomplished by use of a bioerodable polymer non-cellular source of the growth factor or compound, or by co-encapsulating a cellular source of the growth factor or compound, or any other suitable means. See, e.g., U.S. Pat. Nos. 5,106,627 and 5,156,844.

Control of Growth By Irradiation

Cell proliferation can also be controlled through exposure of cells to a suitable dose of irradiation, e.g., x-rays, ultraviolet (UV) radiation, and the like. When cells are subjected to irradiation, their progression through the cell cycle may be arrested. The critical dose rate, or minimum dose rate can be determined for a chosen cell type using methods known in the art. See, e.g., Stanley and Lee, *Radiat. Res.,* 133, pp. 163–9 (1993); Mitchell et al., *Radiat. Res.,* 79, pp. 537–51 (1979). For example, normal human epidermal keratinocytes irradiated with 5 and 10 mJ/cm^2 ultraviolet B(UVB) radiation showed a significant (up to 78%) decrease in proliferation 3 to 5 days post-irradiation. Prystowsky et al., *J. Invest. Dermatol.,* 101, pp. 54–58 (1993). Yi et al., *Radiation Research,* 133, pp. 163–69 (1993) provide a method for calculating the lowest dosage required to stop cell proliferation by exposure to x-rays.

Control of Growth and Differentiation By Use of Extracellular Matrix Molecules

Methods are provided herein for the control of cell distribution in a BAO by modification of a growth surface with a growth controlling extracellular matrix ("ECM") (or components thereof) alone or in combination with a growth controlling physical matrix or other growth regulating substances.

In living tissue, the ECM is formed from a variety of proteins and polysaccharides which are secreted by cells and assembled into a network in proximity to the cells that secreted them. ECM molecules include glycosaminoglycans and proteoglycans, such as chrondroitin sulfate, fibronectin, heparin sulfate, hyaluron, dermatan sulfate, keratin sulfate, laminin, collagen, heparan sulfate proteoglycan (HSPG) and elastin. In particular, collagen is a major component of ECM in vivo. ECM molecules are known to cause decreased cell proliferation and increased cell differentiation. In addition, acellular ECM when used in the methods of this invention may influence the spatial location of cells encapsulated in the BAO.

ECM may be obtained by culturing cells known to deposit ECM, including cells of mesenchymal or astrocyte origin. Schwann cells can be induced to synthesize ECM when treated with ascorbate and cAMP. These ECM components resemble a precursor form of the basement membrane which support Schwann cell proliferation. Furthermore, naturally produced ECM from endothelial cells and a reconstituted basement membrane gel from Engelbreth Holm-Swarm tumor cells (EHS) supports the growth and differentiation of various epithelial and endothelial cells. Baron-Van Evercooren et al., "Schwann Cell Differentiation in vitro: Extracellular Matrix Deposition and Interaction," *Dev. Neurosci.,* 8, pp. 182–96 (1986).

In one embodiment, growth control is achieved by coating a growth surface in the BAO with ECM (or its growth controlling components). We prefer seeding the growth surface in the BAO with cells that produce ECM, and culturing the cells until confluent. The cells are then treated with detergent and NH_4OH. The resulting BAO, with acellular ECM coated on a growth surface, is then used to encapsulate cells that produce the desired biologically active molecule.

In another embodiment, ECM is prepared substantially in the same manner in vitro, lyophilized, fragmented and

mixed with cells as a suspension. The cell/ECM fragments are then co-loaded into the BAO.

Cells grown in presence of some ECM molecules show decreased proliferation and increased differentiation compared to cells grown in conventional monolayer culture. For example, adrenocortical cells, known to synthesize certain steroid hormones such as aldosterone, exhibit decreased proliferation when grown in vitro in the presence of collagen gel. Fujiyama et al., "Influence of Extracellular Matrix on the Proliferation and Differentiation of Adrenocortical Cells in Culture," *Path. Res. Pract.*, 189, pp. 12051–14 (1993).

Schwann cells may also exhibit decreased proliferation and increased differentiation when cultured in the presence of collagen.

Endocrine cells are also known to differentiate in vitro when grown on surfaces coated with a combination of type IV collagen and HSPG. Type IV collagen is necessary for cell adhesion and the HSPG induces differentiation. de Bruine et al., "Extracellular Matrix Components Induce Endocrine Differentiation In Vitro in NCI-H716 Cells," *American Journal of Pathology*, 142, pp. 773–782 (1993).

Various growth factors or chemical compounds, including those discussed supra, may be added to the ECM components to further control the growth and differentiation of cells. Growth factors may be administered to the cells in vitro prior to implantation or to the cells in vivo, or both. See, e.g., U.S. Pat. Nos. 5,156,844 and 5,106,627, which refer to methods for delivering growth factors using either a co-encapsulated cellular or non-cellular source of the growth factor. In addition, the ECM molecules may be derivatized with growth controlling peptides according to known techniques.

For example, transforming growth factor-β, which modulates cell growth on its own, and which reversibly binds to certain ECM molecules (e.g. decorin), can be added to ECM to potentiate the growth-inhibiting effects of ECM molecules.

Likewise, heparin has also been shown to prevent the growth of both untransformed cells and transformed cell lines. Matuoka et al., *Cell Structure and Function*, 9, p. 357 (1984).

Basic fibroblast growth factor (bFGF) has also been reported to enhance endocrine cell differentiation when added along with ECM components. See, de Bruine et al., "Extracellular Matrix Components Induce Endocrine Differentiation In Vitro in NCI-H716 Cells," *American Journal of Pathology*, 142, pp. 773–782 (1993).

Growth factors may exhibit different effects on cells when combined with different components of ECM. For example, fibroblast growth factor (FGF) has been shown to be an effective differentiating factor and a weak mitogen for chromaffin cells grown on laminin. However, when FGF is added to chromaffin cells grown on collagen, FGF is a weak differentiation factor and a strong mitogen. This behavior has also been shown for the cyclic AMP analogue 8-(4-chlorophenylthio) cyclic AMP. Chu et al., *Neuroscience*, 95, pp. 43–54 (1994).

Table 1 is a partial list of ECM molecules growth factors and chemical compounds known to influence proliferation and differentiation in particular cell types.

TABLE 1

ECM MOLECULES, GROWTH FACTORS AND CHEMICAL COMPOUNDS INFLUENCING PROLIFERATION OR DIFFERENTIATION

Cell Type	Differentiation Inducer/ Growth Inhibitor	Proliferation Promoter
Schwann	ascorbate; collagen (Vitrogen ™); Cultisphers/agarose	TGF-β; dbcAMP
PC12	NGF; dbcAMP; SGP	
Fibroblasts	TGF-β-1; Cultisphers/ agarose; ascorbate; SGP	Vitrogen ™
Myoblasts	collagen; ascorbate	
Neural stem	laminin; Peptite 2000; Cultisphers/Peptite 2000; phorbol ester; heparin; FGF and (CNTF or NGF)	EGF; bFGF; TGF-α; amphiregulin
Human embryonal rhabdomyosarcoma cell line	Ara-C	
Human fetal pancreatic islet cells	Nicotinamide (NIC)	
Astroblasts	dbcAMP	
Swiss 3T3	SGP	
Adrenocortical	Collagen	
Endocrine	Type IV Collagen + HSPG; bFGF + ECM components	
Chromaffin	FGF + laminin; 8-(4-chlorophenylthio) cyclic AMP + laminin	FGF + collagen; 8-(4-chlorophenylthio)cyclic AMP + collagen
Hematopoietic stem cells		Mast cell Growth Factor
BHK	TGFβ-1 + Ascorbate; ECM from E15 rat meningeal cells	
Keratinocytes	TGFβ-1	
Endothelial cells	TGFβ-1	
Neuroendocrine (human pancreatic caranoid cells (BON))	TGFβ-1	TGFβ-1 + Ascorbate; Serotonin; FGF
Human neuroblastoma Cell line IMR-32 SCT-1	Mitomycin C + BrdU; PGE_1+ dbcAMP; SGP Collagen; Ascorbate	

The growth surfaces within the BAO include the luminal surfaces of the BAO, and additionally include other growth surfaces, such as an inner support, that may be encapsulated within the BAO.

Microcarriers may provide a surface for cell growth. Use of microcarriers can allow a greater number of cells to be encapsulated and evenly distributed within the BAO, especially for cells that become growth contact inhibited. Several types of microcarriers are commercially available, including Cytodex (Sigma, St. Louis, Mo.) dextran microcarriers, and CultiSpher™ (HyClone Labs, Logan, Utah) macroporous gelatin microcarriers and glass microcarriers. These microcarriers are often used for the culture of anchorage dependent cells. Cell lines which have been shown to grow on macroporous gelatin microcarriers include 0BHK, BHK-21, L-929, CHO-K1, rCHO, MDCK, V79, F9, HeLa, and MDBK. Microcarriers may also be made of or coated with other ECM molecules (such as FACT™ collagen coated microcarriers (Solo Hill Labs, Ann Arbor, Mich.)), or acellular ECM, substantially as described above.

In one preferred embodiment cells producing the desired biologically active molecules can be seeded onto the ECM coated microcarrier surfaces and cultured on the microcarriers in vitro, prior to encapsulation and implantation. Cherksey (WO 93/14790) refers to the culturing of cells on

glass or plastic microbeads and subsequent implantation of the microbeads into the brain of a recipient.

In another embodiment according to this invention, cells seeded on microcarriers may be suspended in the presence of a suitable growth-inhibiting matrix and then encapsulated in the BAO. Such matrix material (e.g., agarose or agar for fibroblasts; collagen for adrenocortical cells) physically inhibits further cell outgrowth. Such hydrogel matrices are described in, e.g., Dionne WO 92/19195, incorporated herein by reference.

According to another aspect of this invention, agarose may also be used as a substitute for ECM by derivatization with peptide sequences to affect cell attachment to the matrix. For example, agarose hydrogels may be derivatized with peptide sequences of laminin or fibronectin.

In this method, cells are suspended in 3-D matrices composed of agarose derivatized with a peptide sequence that recognizes a cell surface receptor molecule involved in cell adhesion. Several peptide sequences have been shown (in 2-D) to promote cell adhesion. See, e.g., Pierschbacher et al., *Science,* 309, pp. 30–33 (1984); Graf et al., *Biochemistry,* 26, pp. 6896–900 (1987); Smallheiser et al., *Dev. Brain Res.,* 12, pp. 136–40 (1984); Jucker et al., *J. Neurosci. Res.,* 28, pp. 507–17 (1991). The derivatized agarose matrices of this invention allow presentation of the appropriate molecular cues for cell adhesion in 3-D. The agarose concentration is preferably 1.25% w/v or less, most preferably about 1.0%. We prefer RGD-containing sequences (i.e. ArgGlyAsp; AA_2–AA_4 of SEQ ID NO:2), YIGSR-containing sequences (TyrIleGlySerArg; AA_5–AA_9 of SEQ ID NO:1), IKVAV-containing sequences (IleLysValAlaVal; AA_{11}–AA_{15} of SEQ ID NO:3), and the like. Derivatization can be achieved using a bi-functional coupling agent, such as 1'1, carbonyldiimidazole or any other suitable method.

One particular advantage of using agarose instead of ECM components is that naturally occurring ECM components may be enzymatically degraded over time in vivo while agarose is not as readily degraded. The use of agarose is also advantageous because it is a defined product unlike materials like Matrigel®, which is derived from a tumor cell line and therefore an undefined mixture. Specifically, it has been shown that Matrigel® contains bFGF, a potent mitogen for many cell types. Agarose is a clear, thermoreversible hydrogel made of polysaccharides. In addition to physically restricting cell outgrowth, agarose itself may inhibit proliferation and induce differentiation. See, e.g., Aulthouse, in "Expression of the Human Chondrocyte Phenotype In Vitro," *In Vitro Cellular & Developmental Biology,* 25, pp. 659–668 (1989).

Agarose can be chemically modified by derivatives, e.g., PEO-PDMS, to further inhibit cell outgrowth, preferably without toxic effects to the cells.

It will be appreciated that different cell types may exhibit different responsiveness to a given ECM molecule, or to acellular ECM from a particular source. See, e.g., End and Engel, "Multidomain Proteins Of The Extracellular Matrix And Cellular Growth", pp. 79–129, in *Receptors For Extracellular Matrix,* [Eds] McDonald and Mecham, Academic Pree, New York (1991), herein incorporated by reference. One of ordinary skill can readily screen a cell type to determine its responsiveness to an ECM molecule or to acellular ECM from a specific source, to determine its effectiveness in controlling cell distribution.

Growth Control by Growth Surface Modification in the BAO

Methods are provided herein for cell growth control in a BAO by chemically modifying growth surfaces to control cell number and cell location within the BAO. Growth surfaces within the bioartificial organ can be modified to control cell attachment to the growth surface. The growth surface within the BAO can be the luminal surface of the BAO, or an internal membrane, microcarrier or inner support placed inside the BAO. With the microcarrier and inner support embodiments, cells can be cultured on these structures in vitro and subsequently encapsulated in the BAO for implantation.

The BAO membrane may be modified by a number of different known methods, including chemical modification, to produce carboxylic acid groups, amine groups, or hydroxyl groups or other reactive functional groups, or it can be modified by absorption. These reactive functional groups, otherwise not present on the polymer backbone, can subsequently be used as sites for further derivatization.

In one embodiment, the luminal surface of the BAO is modified to promote cellular attachment thereto. Controlled cell attachment to the luminal surface may be useful in enhancing cell survival. By attaching the cells preferentially to the membrane, an even distribution of cells inside the capsule can be achieved with fewer cells than that are used in immobilization techniques using a hydrogel suspension. The use of fewer cells results in a lesser amount of cellular debris. Another benefit is the enhanced diffusion of nutrients to the cells because the cells are in close contact with the membrane. If the membrane modification is used without a matrix material within the capsule, complications of transport through the gel and adsorption of proteins or cell products to the matrix material can also be avoided. Cellular attachment may be promoted by treatment of the BAO luminal surface with poly(d-lysine) of various molecular weights. The poly(d-lysine) can be adsorbed onto the BAO luminal surface from a pH 11 buffered solution. We prefer poly(d-lysine) of about 67,000 g/mole.

In addition, peptide derivatives, e.g., RGD containing sequences (ArgGlyAsp; AA_2–AA_4 of SEQ ID NO:2), YIGSR-containing sequences (TyrIleGlySerArg; AA_5–AA_9 of SEQ ID NO:1), including CDPGYIGSR (CysAspProGlyTyrIleGlySerArg; SEQ ID NO:1), as well as IKVAV containing sequences (IleLysValAlaVal; AA_{11}–AA_{15} of SEQ ID NO:3) (preferably CysSerArgAlaArgLysGlnAlaAla SerIleLysValAlaValSerAlaAspArg (SEQ ID NO:3)), have been found to be particularly useful in promoting cellular attachment. For example, RGD (ArgGlyAsp; AA_2–AA_4 of SEQ ID NO:2), the most common of these peptides can be chemically attached to the BAO membrane, using known techniques. Some RGD (ArgGlyAsp; AA_2–AA_4 of SEQ ID NO:2) containing molecules are commercially available—e.g., PepTite-2000™ (Telios).

In another embodiment, the BAO membrane can be modified to inhibit cell attachment through adsorption of, e.g., PEO-PDMS or poly(d-lysine)alginate. We prefer PEO-PDMS modification, particularly if the growth surface is porous. This is because PEO-PDMS will tend to diffuse through the pores and adsorb to the surface as it passes through the pores through hydrophobic-hydrophobic bonding. In particular, low molecular weight (600–3000 g/mole) PEO-PDMS is preferred.

This embodiment is particularly useful when cells are grown on microcarriers and encapsulated in the BAO. In this manner, an even cell distribution may be achieved, cell number may be controlled, and cell adhesion may be limited to the microcarrier.

In addition, compounds promoting and inhibiting cell attachment can be used in combination. For example, the

luminal surface of the BAO can be treated with compounds inhibiting cell attachment, and cell-carrying microspheres, or the matrix surrounding the cells (if used), may be treated with compounds promotimg cell attachment.

In another embodiment, the interior of the BAO may be altered by providing an inert scaffold within the BAO prior to loading cells. This scaffold provides a structure for adhering and evenly distributing cells within the capsule. Compounds useful in the preparation of an inert scaffold include, poly(hydroxyethyl methacrylate) ("PHEMA") and poly(hydroxyethyl methacrylate-co-methyl methacrylate) ("PHEMA/MMA"). Furthermore, the scaffold may be derivatized with various chemicals or proteins, including those discussed supra, to further control growth and differentiation. According to this method, solutions of a suitable scaffold material are precipitated in the BAO for the desired scaffold.

Another embodiment contemplates culturing cells on a member which will serve as an internal support. The internal support may be made of any substantially biocompatible material such as titanium or a suitable polymer. The support can be in the form of a strut or may be designed to also function as a scaffold, by providing a large amount of surface area for cell growth. One example of such a scaffold material is a non-woven polyester fabric (NWPF) (Reemay, Tenn.). There are numerous types of NWPF, varying in tightness of weave and thickness of the sheet. Such technique allows precise control over number of cells in a BAO, as well as the ability to qualify the cells/scaffold prior to insertion in the BAO. Further, differentiation of cells cultured on such a material (external to the device) could be accomplished prior to insertion of the material into the device. Such a scaffold could be modified, for example, with cell adhesion peptides, to induce cellular differentiation. Additionally, the material adds strength to the BAO. The fabrication of BAOs containing an inner support is described in co-pending application Ser. No. 08/105,728

The BAOs useful in this invention typically have at least one semipermeable outer surface membrane or jacket surrounding a cell-containing core. The jacket permits the diffusion of nutrients, biologically active molecules and other selected products through the BAO. The BAO is biocompatible, and preferably immunoisolatory. The core contains isolated cells, either suspended in a liquid medium or immobilized within a hydrogel matrix.

It is to be understood that the foregoing methods and compositions for controlling the distribution of cells within a BAO are not exclusive. It may be desireable to use several of the methods and compositions in combination to achieve the desired growth control.

For example, it may be desirable to produce cells that have been genetically modified to include a growth controlling gene according to the methods of this invention, grow those cell on ECM microcarriers, and encapsulate the cell/microcarrier clusters in a BAO in which one or more growth surfaces have been modified to control cell distribution.

The encapsulating membrane of the BAO may be made of a material which is the same as that of the core, or it may be made of a different material. In either case, a surrounding or peripheral membrane region of the BAO which is permselective and biocompatible will be formed. The membrane may also be constructed to be immunoisolatory, if desired.

The choice of materials used to construct the BAO is determined by a number of factors and is described in detail in Dionne WO 92/19195. Briefly, various polymers and polymer blends can be used to manufacture the capsule jacket. Polymeric membranes forming the BAO and the growth surfaces therein may include polyacrylates (including acrylic copolymers), polyvinylidenes, polyvinyl chloride copolymers, polyurethanes, polystyrenes, polyamides, cellulose acetates, cellulose nitrates, polysulfones, polyphosphazenes, polyacrylonitriles, poly (acrylonitrile/covinyl chloride), as well as derivatives, copolymers and mixtures thereof.

BAOs may be formed by any suitable method known in the art. One such method involves coextrusion of a polymeric casting solution and a coagulant which can include biological tissue fragments, organelles, or suspensions of cells and/or other therapeutic agents, as described in Dionne, WO 92/19195 and U.S. Pat. Nos. 5,158,881, 5,283,187 and 5,284,761, incorporated herein by reference.

The jacket may have a single skin (Type 1, 2), or a double skin (Type 4). A single-skinned hollow fiber may be produced by quenching only one of the surfaces of the polymer solution as it is co-extruded. A double-skinned hollow fiber may be produced by quenching both surfaces of the polymer solution as it is co-extruded. Typically, a greater percentage of the outer surface of Type 1 hollow fibers is occupied by macropores compared to Type 4 hollow fibers. Type 2 hollow fibers are intermediate.

Numerous capsule configurations, such as cylindrical, disk-shaped or spherical are possible.

The jacket of the BAO will have a pore size that determines the nominal molecular weight cut off (nMWCO) of the permselective membrane. Molecules larger than the nMWCO are physically impeded from traversing the membrane. Nominal molecular weight cut off is defined as 90% rejection under convective conditions. In situations where it is desirable that the BAO is immunoisolatory, the membrane pore size is chosen to permit the particular factors being produced by the cells to diffuse out of the vehicle, but to exclude the entry of host immune response factors into the BAO. Typically the nMWCO ranges between 50 and 200 kD, preferably between 90 and 150 kD. The most suitable membrane composition will also minimize reactivity between host immune effector molecules known to be present at the selected implantation site, and the BAO's outer membrane components.

The core of the BAO is constructed to provide a suitable local environment for the particular cells isolated therein. The core can comprise a liquid medium sufficient to maintain cell growth. Liquid cores are particularly suitable for maintaining transformed cell lines like PC12 cells. Alternatively, the core can comprise a gel matrix. The gel matrix may be composed of hydrogel (alginate, "Vitrogen™", etc.) or extracellular matrix components. See, e.g., Dionne WO 92/19195.

Compositions that form hydrogels fall into three general classes. The first class carries a net negative charge (e.g., alginate). The second class carries a net positive charge (e.g., collagen and laminin). Examples of commercially available extracellular matrix components include Matrigel™ and Vitrogen™. The third class is net neutral in charge (e.g., highly crosslinked polyethylene oxide, or polyvinylalcohol).

Any suitable method of sealing the BAO may be used, including the employment of polymer adhesives and/or crimping, knotting and heat sealing. These sealing techniques are known in the art. In addition, any suitable "dry" sealing method can also be used. In such methods, a substantially non-porous fitting is provided through which the cell-containing solution is introduced. Subsequent to filling, the BAO is sealed. Such a method is described in copending U.S. application Ser. No. 08/082,407, herein incorporated by reference.

One or more in vitro assays are preferably used to establish functionality of the BAO prior to implantation in vivo. Assays or diagnostic tests well known in the art can be used for these purposes. See, e.g., *Methods In Enzymology*, Abelson [Ed], Academic Press, 1993. For example, an ELISA (enzyme-linked immunosorbent assay), chromatographic or enzymatic assay, or bioassay specific for the secreted product can be used. If desired, secretory function of an implant can be monitored over time by collecting appropriate samples (e.g., serum) from the recipient and assaying them. If the recipient is a primate, microdialysis may be used.

The number of BAOs and BAO size should be sufficient to produce a therapeutic effect upon implantation is determined by the amount of biological activity required for the particular application. In the case of secretory cells releasing therapeutic substances, standard dosage considerations and criteria known to the art are used to determine the amount of secretory substance required. Factors to be considered are discussed in Dionne, WO 92/19195.

Implantation of the BAO is performed under sterile conditions. Generally, the BAO is implanted at a site in the host which will allow appropriate delivery of the secreted product or function to the host and of nutrients to the encapsulated cells or tissue, and will also allow access to the BAO for retrieval and/or replacement. The preferred host is a primate, most preferably a human.

A number of different implantation sites are contemplated. These implantation sites include the central nervous system, including the brain, spinal cord, and aqueous and vitreous humors of the eye. Preferred sites in the brain include the striatum, the cerebral cortex, subthalamic nuclei and nucleus Basalis of Meynert. Other preferred sites are the cerebrospinal fluid, most preferably the subarachnoid space and the lateral ventricles. This invention also contemplates implantation into the kidney subcapsular site, and intraperitoneal and subcutaneous sites, or any other therapeutically beneficial site.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of this invention in any manner.

EXAMPLES

Example 1

Growth Control Using the Mx1 Promoter

The mouse Mx1 promoter was fused with the SV40 early region and the chimeric gene was used to generate transgenic mice. Because the Mx1 promoter elements are induced in the presence of IFNα or IFNβ, oncogene expression in tissues or in cell cultures prepared from the transgenic animals can be controlled. Thus, conditionally-immortalized cell lines can be generated.

Production Of Transgenic mice

The Mx1-Tag construct we used consisted of approximately 2 kb of the Mx1 promoter (i.e., Xba1-EcoR1 fragment) fused to an intact SV40 early region cDNA, which encodes both large T and small T antigens and is fused upstream of the mouse beta globin 3' untranslated region and poly-A signal (BamH1-Xba1 fragment). The beta globin sequences were included to provide splice sites and to enhance expression of the cDNA in transgenic animals. FIG. 1 shows the plasmid map of the Mx-1 construct.

Transgenic mice containing the Mx1-Tag construct were produced by the standard technique of pro-nuclear microinjection into single-cell fertilized mouse ova (Brinster et al., *Proc. Natl. Acad. Sci. USA*, 82, pp. 4438–4442 (1985)). Southern blot analysis of tissues from the founder animals confirmed that intact copies of the transgene were integrated in the genome.

Offspring from these mice were confirmed as "DNA positive" using PCR amplimers that recognize sequences of the SV40 early region.

Conditionally-Immortalized Stem Cells

Striata were removed from E15 transgenic mouse embryos and DNA negative littermates and plated in primary (individual) cell culture in EGF-containing neurospheres medium (per 100 mls: DDH_2O 50 ml, 10× DMEM/F12 10 ml, 30% glucose 2.0 ml, $NaHCO_3$ 1.5 ml, 1M HEPES 0.5 ml, L-glutamine 1.0, 10× hormone mix 10 ml, DDH_2O 25 ml (to wash filter)). Neurospheres were prepared according to the method of Weiss, PCT CA92/00283, and Reynolds and Weiss, *J. Neuroscience*, 12, pp. 4565–74 (1992). Cells were passaged seven times once a week and then divided into 2 groups: with and without exogenous interferon (IFN). Cells were placed in T25 flasks at a plating density of 500,000 cells/5 ml in EGF-containing neurosphere medium. 1000 units/ml IFN were added to ½ of the cells. Control neurospheres received no IFN. The cells were incubated at 37° C., 5% CO_2 and were passaged weekly.

After 30 passages (23 with IFN), the cells were placed in serum-containing medium (DMEM, 5% fetal bovine serum, and 1× L-glutamine) with 1000 units of IFN at a cell density of 1.25 million cells in 15 ml. Fresh IFN was added every other day.

Seven days later, the medium was removed, the cells were washed with Hanks' Balanced Salt Solution (HBSS), and the flask was lightly trypsinized. The cells were resuspended in 10 ml of the serum-containing medium, spun down at 1000 RPM for 2 minutes, and the medium was aspirated off. The cells were then resuspended in 2 ml of serum medium by triturating with a fire-polished pipet.

Approximately 25,000 cells were plated on poly-ornithine-treated coverslips in DMEM with 5% FBS. IFN was added to half of the coverslips (1000 units/ml) every other day. Cells were stained for SV40 T-antigen (Tag) and glial fibrillary acidic protein (GFAP), an intermediate filament protein specifically expressed in astrocytes, at various intervals, according to the following protocol.

Coverslips were immersed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) for 20 mins. at room temperature, and then washed twice for 5 mins. in PBS. Cells were permeabilized in 100% EtOH for 2 min, and then washed again twice for 5 min. in 0.1 M PBS. Cells were blocked with 5% NGS (normal goat serum) diluted in 0.1 M PBS for at least 30 mins at room temperature. Primary antibodies were pooled and diluted in 1% NGS for 2 hrs. and were applied to the coverslips at room temperature, as follows: anti-Tag (mouse monoclonal) was diluted 1:10, anti-GFAP (rabbit polyclonal) was diluted 1:500. The primary antibodies were removed and the coverslips were then washed twice for 5 mins. with PBS.

Secondary antibodies were pooled and diluted in 1% NGS and were applied to the coverslips for 30 min. at room temperature in the dark, as follows: GAM-FITC (1:128); GAR-Texas Red (diluted 1:100). The secondary antibodies were removed and the coverslips were washed twice for 5 mins. with PBS in the dark.

The coverslips were mounted with Citifluor™ (or other anti-fadent mounting media) onto slides and stored at 4° C. until viewing using a fluorescent microscope equipped with rhodamine and fluorescein optics.

In this set of experiments, we set out to determine how quickly T-antigen levels fall upon the removal of the interferon. In addition, we were interested to determine the effect of T-antigen level on cell proliferation and differentiation. Differentiation was assessed by monitoring GFAP level. GFAP is an intermediate filament protein specifically expressed in mature astrocytes. The following immunofluorescence results were observed.

	IFN (1000 units/ml)		Control (No IFN)	
Day	Tag	GFAP	Tag	GFAP
1	+++	–	+++	–
4	+++	–	+	+/–
7	+++	–	+/–	+/–
10	+++	–	–	+

Thus, as shown by Tag and GFAP immunostaining, after a period of time in the serum medium, the IFN-treated cells showed continued expression of T-antigen, continued proliferation, and no evidence of GFAP expression, while the controls (no IFN) began to differentiate (upregulated GFAP expression) and ceased dividing. This was confirmed by a visual inspection of the coverslips—there was a clear cut difference in cell numbers by day 4. By day 10, the IFN-treated cells were much more numerous than the controls.

The expression of the SV40 T-antigen in this construct is regulated in a dose-dependent manner. In the cell lines we have produced, maximal T-antigen expression (measured by immunofluorescence) was observed at an IFN dose of 500–1000 units/ml. At 100 units/ml we observed minimal to no expression. As would be expected, the rate of proliferation correlated with the IFN dose; there was little or no cell division at 100 units/ml of IFN.

Example 2

Cells prepared according to Example 1 are encapsulated and implanted in a human host.

Preparation Of PAN/PVC Fibers

Permselective hollow fibers are prepared via a dry jet-wet spinning technique (Cabasso, *Hollow Fiber Membranes,* vol. 12, *Kirk-Othmer Encyclopedia of Chemical Technology,* Wiley, N.Y., 3rd Ed., pp. 492–517, 1980; Dionne, WO 92/19195; U.S. Pat. No. 5,158,881). Asymmetric hollow fibers are cast from solutions of 12.5% polyacrylonitrile polyvinyl chloride (PAN/PVC) copolymer in dimethyl sulfoxide (w/w). Single-skinned or double-skinned fibers are produced. The fibers are collected into a non-solvent water bath, glycerinated, and dried. Cells are loaded at a density of 25,000 cells/μl into a PAN/PVC single-skinned hollow fiber and sealed by heat pinching.

Implantation Into Host

The encapsulated cells are implanted into a human host. Implantation sites include the lateral ventricles and striatum of the brain. Procedures for implantation of BAOs into the brain are described in Aebischer et al., WO 93/00127, incorporated herein by reference.

Example 3

Conditional Immortalization of Neonatal Astrocytes

A fragment containing the promoter elements of mouse mammary tumor virus (MMTV) is fused to the SV40 early region cDNA. E15 rat brain derived neonatal astrocytes are transfected by electroporation and transformants selected by assaying for proliferation. Dividing cells are removed, expanded and assayed for expression of large T-antigen, using anti-large T antibodies. Transformed cells are encapsulated in BAOs and implanted in a host, substantially as described in Example 2. The BAOs are held in vivo for one month. The BAOs are then retrieved and the cell distribution in the BAOs compared to cohorts held in vitro for the same time period.

Example 4

Collagen-Reduced Proliferation and Ascorbate-Induced Differentiation Of SCT-1 Cells

SCT-1 cells were cloned from a sciatic nerve tumor from a Po-SV40 transgenic mouse (Messing et al., *J. Neuroscience,* 14, pp. 3533–39 (1994). These SCT-1 cells were immunoreactive for the Schwann cell markers S100 and Po, as well as for SV40 T-antigen.

SCT-1 cells were grown under three conditions: (1) on tissue culture plastic without ascorbate, (2) on tissue culture plastic in the presence of 50 μg/ml ascorbate to induce differentiation, and (3) suspended in Type I collagen.

On a plastic substratum in the absence of ascorbate, most cells displayed a fibroblast-like morphology. However, some bipolar cells were present. Cells doubled in 18–20 hours and displayed no contact inhibition.

SCT-1 cells grown in the presence of ascorbate demonstrated slower growth and a more robust staining for fibronectin and type IV collagen. Laminin immunoreactivity, on the other hand, was similar in control and ascorbate-induced differentiated cultures.

SCT-1 cells suspended in Type I collagen exhibited a bipolar morphology and a dramatic decrease in mitotic activity (i.e., doubling time was ≧30 days).

Example 5

Inhibition of BHK Cell Proliferation By Ascorbate and TGF-β

BHK cells secreting CNTF were grown in DMEM (high glucose) medium. Treatment of subconfluent BHK cultures with TGF-β1 (2.5 ng/ml) and ascorbate (100 μM) reduced mitosis. In addition, the cells appeared elongated, with some cells aligning. This data indicates TGF-β1 and ascorbate inhibits proliferation and induces differentiation of BHK cells.

In further experiments, BHK cells secreting hNGF were treated with 2.5 ng/ml TGFβ and 100 μM ascorbic acid prior to encapsulation in BAOs and implantation. Non-treated cells served as controls. The specific variables include: a) TGFβ/ascorbate, no Vitrogen™, b) TGFβ/ascorbate, Vitrogen™, c) no TGFβ/ascorbate, Vitrogenl™, and d) no TGFβ/ascorbate, no Vitrogen™. In addition, several different polymers were used. Capsules were implanted into the striatum of adult rats. Rats were sacrificed after 3 mos.

Example 6

Neural Stem Cells Proliferate in the Presence of EGF and Differentiate in its Absence

Neurospheres were prepared using the methods of Weiss et al., PCT/CA 92/00283. Passage 68 neurospheres were collected and divided. Half of the neurospheres were triturated into a single cell suspension and half remained as

clusters. A single cell count was performed on a single cell suspension and it was assumed that the clustered cells were of the same concentration. Single cells and clusters were suspended separately in equal amounts of Vitrogen™ and either neurosphere medium with 20 ng/ml EGF as controls, or PC-1 medium.

Cells were loaded at a density of 25,000 cells/μl into single-skinned hollow fiber PAN/PVC BAOs, prepared substantially as described in Example 2, and then hub sealed. The BAOs were held in either neurosphere+EGF medium or in PC-1 medium (with no EGF).

The BAOs were sacrificed after 3 days and 7 days and were stained for glial fibrillary acidic protein (GFAP) by immunocytochemistry. GFAP is an intermediate filament protein specifically expressed in astrocytes. GFAP reactivity indicates that the neural stem cells have differentiated into astrocytes. The following results were observed:

	Time (days)	GFAP Reactivity
Single cell, no EGF	3	Small % + for GFAP
Single cell, EGF	3	Negative
Cell clusters, no EGF	3	Small % + for GFAP
Cell clusters, EGF	3	Negative
Single cell, no EGF	7	Intense + for GFAP
Single cell, EGF	7	Negative
Cell Clusters, no EGF	7	Intense + for GFAP
Cell Clusters, EGF	7	Negative

By day 7, the encapsulated neural stem cells had differentiated into astrocytes in the absence of EGF.

Example 7

Effect of ECM on BHK Cells

Preparation Of Acellular ECM

E15 rat meningeal cells obtained from 15 day old embryonic rats were plated in multiwell plates and allowed to become confluent. The cells were monolayer contracted after 2 weeks and were allowed to regrow.

Acellular ECM was extracted by treatment with 0.1% Triton X-100 detergent for 30 mins, and then treatment with 5 mM NH_4OH for 3 mins.

BHK-hNGF Cells

A BHK cell line secreting NGF was produced as follows. A 2.51 kb fragment containing approximately 37 bp of the 3' end of the first intron, the double ATG sequence believed to be the protein translation start for pre-pro-NGF and the complete coding sequence and entire 3' untranslated region of the human NGF gene (Hoyle et al., *Neuron,* 10, pp. 1019–34 (1993)) was subcloned into the DHFR-based pNUT expression vector immediately downstream from the mouse metallothionein-1 promotor (−650 to +7) and the first intron of the rat insulin II gene (Baetge et al., *Proc. Natl. Acad. Sci.,* 83, pp. 5454–58 (1986)).

Baby hamster kidney (BHK) cells were transfected with the pNUT-βNGF construct using the calcium phosphate method. BHK cells were grown in DMEM containing 10% fetal bovine serum, 1xpenicillin/streptomycin/ampicillin B (0.8 g/l), and L-glutamine (GIBCO) in 5% CO_2 and at 37° C. Transfected BHK cells were selected in medium containing 200 μM methotrexate (Sigma) for 3–4 weeks and resistant cells were maintained as a polyclonal population either with or without 200 μM methotrexate.

The transformed BHK-hNGF cells were plated at a density of 1.0×10^4 cells/well in the plates containing extracted ECM from meningeal cells. BHK-hNGF cells were also plated at the same density in control plates not containing ECM. Cells were counted using a hemacytometer after 6 DIV.

Cell counts for the control wells averaged $4.5\times10^6\pm4.5\times10^5$ cells. The cell counts for the extracted ECM plates averaged $9.9\times10^5\pm4.9\times10^5$ cells. These results show a 4.5 fold decrease in cell growth on the treated plates.

Example 8

Adherence of Cells To Acellular ECM on an Inner Support

In further experiments, primary menigeal cells were seeded onto a TECO™ polyurethane fiber. Such fibers are useful as inner supports in BAOs. DMEM supplemented with 10% FBS was used as the culture medium. After 2 weeks, the fibers were extracted with 0.1% Triton X-100 for 30 minutes, followed by 25 mM NH_4OH for 3 mins. Some fibers were immunostained with antifibronectin antibody to confirm the presence of acellular ECM on the fiber. Other fibers were used in a cell adhesion assay with BHK cells.

Example 9

BHK Cell Growth on Microcarriers Encapsulated in BAOs Modified With PEO-PDMS

Preparation Of PEO-PDMS Derivatized BAOs

Single-skinned PAN/PVC hollow fiber BAOs were produced as described in Example 2. These BAOs had an ID of 642.6±36.7 μm, an OD of 787.8±32.2 μm, a wall thickness of 67.8±16.2 μm, a BSA rejection coefficient of 100%, and a hydraulic permeability of approximately 21.8 ml/min/m^2/mmHg.

The PAN/PVC BAOs were derivatized with PEO-PDMS under sterile conditions. A 1% or 5% (v/v) solution of PEO-PDMS (Huls, PS073, MW=3126 g/mole; 82% PEO by weight) was prepared by diluting 1 ml or 5 ml of PEO-PDMS to 100 ml with deionized water. The solution was sterile filtered (0.2 μm) prior to injection into a "wet" PAN/PVC membrane. The membrane was heat pinched and immersed in an aqueous solution. The fibers were rinsed with Hanks' Buffered Salt Solution after 72 hrs and prior to use with cells.

NGF-secreting BHK cells as described in Example 7, were loaded into the PEO-PDMS derivatized fibers as follows.

Loading And Sealing Procedure

Single cell suspensions of NGF-producing BHK cells grown to 90% confluency were rinsed with PBS (lacking calcium and magnesium), trypsinized for approximately 1 minute and pelleted by centrifugation at 1000 rpm for 3 minutes. The cells were resuspended in medium to a final cell concentration of 2×10^7 cells/ml.

Cells were either loaded directly into the PEO-PDMS derivatized fibers, or mixed with a 0.15% Vitrogen® matrix solution or 0.5% agarose solution, and then loaded. Approximately 2.5 microliters (ul) of cells or cell/matrix slurry (10,000 cells/ul) were loaded into each fiber using a 24-gauge beveled catheter tip and a Hamilton syringe.

Capsules were sealed by mounting a 1–1.1 cm length of dry hollow fiber onto a hub with a septal fixture at the proximal end which has loading access for cells to be injected into the lumen of the device. After infusing 2.5 μl of the cellular suspension, the septum was cracked off and

the access port sealed using a light-cured acrylate (Luxtrak™ LCM 24, ICI Resins US, Wilmington, Mass.) ("hub" sealed). The capsules were subsequently "tethered" by placing a 1.5 cm 0.020" silastic tube over the acrylic hub.

The following BAOs were prepared in this manner:

1. control underivatized jacket, no matrix;
2. control underivatized jacket, Vitrogen® matrix;
3. control underivatized jacket, agarose matrix;
4. 1% PEO-PDMS derivatized jacket, no matrix;
5. 1% PEO-PDMS derivatized jacket, Vitrogen® matrix;
6. 1% PEO-PDMS derivatized jacket, agarose matrix;
7. 5% PEO-PDMS derivatized jacket, no matrix;
8. 5% PEO-PDMS derivatized jacket, Vitrogen® matrix;
9. 5% PEO-PDMS derivatized jacket, agarose matrix;

The BAOs were maintained at ambient O_2 for 4 days after encapsulation, and then maintained at low O_2 levels (50 mmHg) for the duration of the study. FIG. 2 shows NGF secretion (measured by ELISA) after 4, 11 and 25 days.

The NGF release data indicates that the matrix alone has little effect on the output of the cells. However, in the presence of PEO-PDMS, the NGF release is substantially lower when used with agarose and without a matrix but not affected by when used with Vitrogen®. In addition, the percent of PEO-PDMS used in the modification apparently had little effect on NGF release. From the histology data, the BHK cells encapsulated with agarose had an elongated morphology and lined the walls of the device; however, very few cells were viable within the agarose itself. The BHK cells loaded with agarose in PEO-PDMS-modified fibers also lined the inner luminal surface of the capsule but had a round morphology. There were fewer cells in the PEO-PDMS-PAN/PVC modified fibers than there were in the unmodified fibers with agarose, indicating that cell growth was controlled. The cells in Vitrogen® loaded devices were not affected by the fiber modification neither were those encapsulated without a matrix.

BHK cells in unmodified fibers with a Vitrogen® matrix were well distributed with approximately 75% viability. There was some cell necrosis in the center of the device. PEO-PDMS modification did not affect cell distribution, viability or morphology. With agarose as the matrix, cell distribution was excellent with cell viability approximating 90%. The cell morphology of BHK cells was affected by PEO-PDMS derivatization of the membrane (1% and 5%) when an agarose matrix was used. The cells were elongated in unmodified P(AN/VC) and more rounded in modified P(AN/VC). Cells were not located in the agarose matrix, but in a space between the fiber and agarose "rod". Without a matrix, the cell distribution is less satisfactory as cells have formed large clusters and the viability is lower (approximately) 60%.

Example 10

BHK Cell Growth On CultiSphers™

NGF-secreting BHK cells as described in Example 7 were grown on collagen coated CultiSphers™. CultiSphers™ (1 g) were rehydrated in 50 ml of PBS (CMF). 15×10^6 cells were suspended in 1 ml of rehydrated CultiSphers™. The cell/CultiSphers™ suspension was loaded directly into single-skinned PAN/PVC hollow fibers, or mixed in a 1:1 ratio with 1% agarose, and then loaded into single-skinned PAN/PVC hollow fibers. The fibers were prepared substantially as described in Example 2, and loaded and sealed substantially as described in Example 9.

The encapsulated cells were tested for NGF secretion by ELISA at 2, 15, and 56 days. The medium was replenished 3 times/week. FIG. 3 shows the results. The NGF release data indicate that BHK cells can grow on CultiSphers™ microcarriers when encapsulated in BAOs (FIG. 3, legend: n-mat-008, 0709-n-m). Further, the NGF release data indicate that BHK cell/CultiSphers™ can be further suspended in an agarose matrix, with little or no effect on NGF secretion (FIG. 3, legend: agaro-008, agaro-0709).

Example 11

Use of a Peptide Derivative to Control Cell Number and Cell Distribution

In this example, the luminal surface of the BAO was modified with PEO-PDMS, poly(d-lysine), or PepTite 2000™, a commercially available cell adhesion protein.

In this study baby hamster kidney (BHK) cells were used because they are anchorage-dependent cells and have been shown previously to adhere to the hollow fiber membrane.

Fibers

Single-skinned PAN/PVC BAOs were produced substantially as described in Example 2. The fiber dimensions were 625 μm ID, 50 μm wall thickness. These fibers were sterilized by immersion in 70% ethanol overnight and then rinsed repeatedly with HBSS.

Derivatization

1. PDMS-PEO: BAOs were derivatized with PDMS-PEO as follows. A 1% (v/v) solution of PEO-PDMS (purchased from Huls, PS073, Mw=3126 g/mole; 82% PEO by weight) was prepared by diluting 1 ml of PEO-PDMS to 100 ml with deionized water. The solution was sterile filtered (0.2 μm) prior to injection into a sterile membrane. The membrane was immersed in a 1% PEO-PDMS aqueous solution for 24 h at room temperature. The fibers were rinsed with water (3 times) and then HBSS prior to injection of cells.

2. PdL: BAOs were derivatized with poly(d-lysine) as follows. Fibers were immersed in an aqueous solution of 67,000 molecular weight poly(d-lysine) at 2 mg/ml for 24 h at room temperature. The fibers were rinsed 3 times with water and then 3 times with HBSS prior to injection of cells.

3. PepTite 2000™: BAOs were derivatized with PepTite 2000™ as follows. Fibers were immersed in a PBS solution of 100 mg/ml of PepTite 2000™ previously dissolved in ethanol. The fibers were immersed in this solution for 24 h at room temperature and then rinsed 3 times with PBS prior to injection of cells.

4. PAN/PVC: Control fibers were immersed in HBSS for 24 h at room temperature prior to injection of cells.

Cells

BHK cells were loaded into the derivatized fibers at a concentration of 5000 cells/μl. The fibers were sealed and placed in screw-cap tubes containing serum-free medium (PC1 medium) and then placed on a rotating drum for up to two weeks in an incubator set at 37° C. The drum speed was 2 rpm. At the appropriate time the fibers were fixed in 4% paraformaldehyde, dehydrated in graded ethanol and stained with hematoxylin and eosin (H&E) for histological analysis of cell distribution with osmium tetraoxide.

PAN/PVC-derivatized membranes showed a good distribution of cells when derivatized with poly(d-lysine) and a more even distribution of cells when derivatized with PepTite™ 2000, as determined by osmium tetroxide staining.

For PAN/PVC membranes, PepTite 2000™ modifications were attempted in two ways. First, the inner luminal surface of the membranes was modified only and second, both the inner luminal surface and the outer surface were treated.

Empty BAOs (i.e. free of cells) were analyzed for total amino acids, to determine the binding of poly(d-lysine) or PepTite 2000™. The total amino acid bound to control, unmodified membranes was approximately 0.2 μg/BAO. The total amino acid bound to poly(d-lysine)-modified membranes was approximately 0.8 μg/BAO for modified inner luminal surface membranes, and approximately 2.6 μg/BAO for membranes where both the inner luminal surface and outer surface had been modified. Similar BAOs loaded with BHK cells were maintained for 14 days, and then examined histologically. In control unmodified BAOs, cells were unevenly located in large clusters over the entire length of the fiber. In contrast, in both types of modified fibers, there was an even distribution of cells along the luminal surface of the membrane.

These results suggest that poly(d-lysine) and PepTite 2000™ are effective in promoting cell attachment to the BAO luminal surface, and thus are effective in controlling cell distribution within the BAO.

Example 12

Use of ECM Molecules to Control Growth of Neurosperes

Passage 71 mouse neurospheres were prepared substantially as in Example 1. Multi-well dishes were precoated with 0.5% agarose (Sea-Prep™) to keep the neurospheres from attaching to the plastic dishes. Cells were plated at a density of approximately 50,000 cells per well into the designated matrices for the experiment. Three wells were used for each matrix condition; two of the wells contained PC-1 medium (control) and one contained neurospheres+ EGF medium(EGF).

A dermal-derived Type 1 collagen (Zydast™; (Collagen Biomedical, Palo Alto)), a tendon-derived Type 1 collagen (Organogenesis™), a Type 1 collagen (Vitrogen™, Celtrix, Santa Clara), and agarose were evaluated for effectiveness in controlling cell growth, alone, or in combination with laminin or PepTite 2000™, or both.

At 4 days and 14 days cells were assayed by staining with fluorescein diacetate/propidium iodide (FDA/PI), and were evaluated for cell viability, growth, and differentiation. Cells exposed to a combination of the Organogenesis™ collagen, Peptite 2000™ and laminin showed the highest amount of differentiation, with about 90% of the cells having undergone differentiation. About 80% of cells exposed to a combination of agarose, Peptite 2000™ and laminin had differentiated.

Example 13

Use of an Inert Scaffold to Control BHK Cell Number and Cell Distribution in a BAO

Two types of PAN/PVC fibers (substantially as described in Example 2) were used: a single-skinned fiber having the permselective membrane on the outer surface, and a single-skinned fiber having the permselective membrane on the inner surface.

First, PAN/PVC fibers were deglycerinized and sterilized by immersion in 70% sterile filtered ethanol overnight. The fibers were then rinsed with sterile water three times over the course of about 1 to 2 hours.

Next, a 15% concentration poly(hydroxyethyl methacrylate) ("PHEMA") scaffold matrix was prepared by dissolving 1.5 g PHEMA in 10 ml of 95% ethanol (190 proof, Quantum). In addition, a 10% concentration poly (hydroxyethyl methacrylate-co-methyl methacrylate) ("PHEMA/MMA") scaffold matrix was made by dissolving 1.0 g of PHEMA/MMA in 10 ml of 95% ethanol. To dissolve the polymers more easily, the solution was stirred and heated.

The PHEMA or PHEMA/MMA solutions were loaded with a syringe into the PAN/PVC fibers, which were then immersed in sterile water. The loaded fibers were left in water for more than 1 hour to ensure precipitation of the scaffolds and diffusion of ethanol out of the core. The ends of the fibers were cut off because they were often clogged with either PHEMA or PHEMA/MMA. The fibers were transferred to Petri dishes containing sterile HBSS. BAOs loaded with PHEMA, PHEMA/MMA and control BAOs were prepared in this manner.

NGF-secreting BHK cells (described in Example 7) were grown in 10% DMEM with glutamine and antibiotics added. The cells were gently pulled off the flasks with 0.25% trypsin, washed and resuspended in PC1 media to a density of 1×10^7 cells/ml.

The BHK-NGF cells were loaded into the fibers at a density of 10,000 cells/μl using a 22 gauge Teflon catheter. BAOs were sealed by heat pinching.

Five BAOs of each type were prepared. Four were placed in a 24 well plate with 1 ml of PC-1 media. The fifth was placed in approximately 3–4 ml of PC-1 media in a vertical tube. After 24 hours, the BAOs placed in the vertical tube were cut open along the lumen (longitudinal cross-section) and analyzed after 24 hours by staining with fluorescein diacetate/propidium iodide (FDA/PI) for cell distribution within the fibers. When viewed under a fluorescent microscope, FDA stains viable cells green and PI stains non-viable cells red.

The remaining BAOs were cultured for 2 weeks. The BAOs were maintained at ambient O_2 for 4 days after encapsulation, and then maintained at low O_2 levels (50 mmHg) for the duration of the study.

The functionality of BHK-NGF cells was tested by measuring NGF secretion (by ELISA) after 4, 7 and 14 days. The cells PHEMA or PHEMA/MMA scaffold-containing BAOs continued to secrete NGF over the duration of the study. Both the histology and NGF-release data indicate that PHEMA and PHEMA-MMA scaffolds allow maintenance of functionally-active viable cells distributed along the BAO. The results with 10% PHEMA-MMA scaffolds were the best.

Example 14

Use of an Inert Scaffold to Control PC12A Cell Number and Cell Distribution in a BAO

The effectiveness of PHEMA and PHEMA/MMA inert scaffolds were evaluated for effectiveness in controlling the distribution of PC12 in BAOs.

Single-skinned fibers were prepared substantially as described in Example 2. These fibers typically had the following characteristics: 642 μm ID, 787 μm OD, wall thickness 68 μm, rejection coefficient 100% (BSA), hydraulic permeability 22 ml/min/m^2/mm Hg.

Inert scaffolds of PHEMA and PHEMA/MMA were prepared in these fibers, substantially as described in Example 13.

PC12A cells (1×10^7 cells/ml) in HL-1 medium were injected into the lumens of the fibers, and the fibers heat sealed to produce BAOs approximately 1 cm long. The

devices were held at 37° C. at ambient pressures in HL-1 media. To assess functionality of the encapsulated cells, the BAOs were tested for basal and K^+-evoked catecholamine release at 1, 14 and 28 days. The results are shown in FIGS. 4A and 5A (basal release) and FIGS. 4B and 5B (K^+-evoked release). These results show that PC12 cells encapsulated in BAOs having inert PHEMA and PHEMA/MMA scaffolds retain their functionality, as measured by catecholamine release.

Cell distribution in the BAOs was evaluated after 5 hours and 4 days by vertically cutting the fibers in half, and staining the cells with FDA/PI. These results indicated that PHEMA and PHEMA/MMA scaffolds are nontoxic and support cell viability and functionality of PC12 cells.

Example 15

Use of an NWPF to Promote Cell Adhesion and Differentiation in a BAO

Six types of NWPF (Reemay, Tenn.) were tried: #2470, #2295, #2024, #2055, #2033, #2250 (Reemay #s). The fabric received was in flatsheet form: discs were punched out to fit into 24 well plates. The NWPF discs were immersed in 1% sodium dodecyl sulphate (SDS), w/v for 6 h and then rinsed with water (3 times). The discs were then immersed in 1% sulfuric acid (v/v in H_2O) for 13 h (overnight) and then rinsed 3 times with water. The discs dried on a paper towel and then sterilized by autoclaving.

The discs were cultured with 3 cell types to test for cell adhesion: BHK, AT-3, and TSA cells. Approximately 100,000 cells were added to a 24 well plate containing one of the above 6 NWPF discs in PC1 media. A serum-free medium was used to test for cell adhesion without the inference of serum (except for TSA cells). After 4 days, the BHK and AT-3 cells were examined for adhesion by PDA/PI. The cells had an elongated morphology and appeared to adhere on Reemay #2250, and 2055. At 10 days, BHK were growing best on #2250. AT-3 cells best adhered to 2024 and 2295. AT-3 cells grew best on 2024 at 10 days. TSA cells (in 10% FCS) after 1 day had an elongated morphology when grown on #2250, #2055, and grew best on #2024. At 7 days, TSA cells were growing best on #2055.

Example 16

SV40/DBH-NGF Cells on Microcarriers Suspended in Matrix Material

Regulatory elements of the dopamine β-hydroxylase (DBH) gene (Hoyle et al., *J. Neurosci.*, 14, pp. 2455–63 (1994)) were utilized to direct the coexpression of the SV40 T-antigen (tsa58) (DBH-SV) and human growth factor (DβH-hNGF) in transgenic mice. Coexpression of the chimeric genes resulted in neoplasms in the adrenal medulla and noradrenergic sympathetic ganglia. A tumor of the celiac region from one of these mice was dissected and the tumor tissue was mechanically dissociated and placed in cell culture (DMEM, 10% FBS, 37 C., 5% CO_2). Two distinct cell types, large flat fibroblast-like cells and small phase-bright cells having extensive neurite processes, were present from the initial culture period. The small cells exhibited features of catecholaminergic neuron including immunoreactivity for neurofilament-L and -M and tyrosine hydroxylase. Immunoreactivity for the SV40 T-antigen was also present in these cells, in contrast to the fibroblast-like cells, which were negative for these markers. The cells were passaged weekly.

Cells were grown on an CultiSphers™ as described in Example 10, and were suspended in either an alginate (1.5%) or agarose (1%) matrix. In the case of the alginate matrix, the alginate was cross-linked by immersing the devices in a 1% aqueous calcium chloride solution for 5 minutes after encapsulation. The cells/Cultisphers™/matrix were loaded into PAN/PVC hollow fibers as described in Example 10.

The cell-loaded BAOs were maintained in serum-free medium conditions. At selected time intervals, devices were washed prior to 30 minute incubations in HBSS. The basal medium was collected and assayed by HPLC-ED for L-dopa. The devices continued to secrete L-dopa at 80 days in vitro.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 4

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Asp Pro Gly Tyr Ile Gly Ser Arg
1 5

-continued

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Arg Gly Asp Ser Pro
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys Ser Arg Ala Arg Lys Gln Ala Ala Ser Ile Lys Val Ala Val Ser
1 5 10 15

Ala Asp Arg

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Gly Gly Gly Gly
1 5

We claim:

1. A method for controlling distribution of cells within a bioartificial organ comprising exposing at least one growth surface within the bioartificial organ to a treatment that inhibits cell proliferation or promotes cell differentiation, wherein the treatment comprises coating the growth surface with an effective amount of at least one extracellular matrix molecule, the extracellular matrix molecule coating formed by culturing extracellular matrix molecule-forming cells on the growth surface and then lysing the cells extracellular matrix molecule-forming cells to produce a coating of at least one acellular extracellular matrix molecule on the growth surface.

2. The method according to claim 1, wherein the growth surface coated with the extracellular matrix molecule comprises an inner luminal surface of the bioartificial organ.

3. The method according to claim 1, wherein the growth surface coated with the extracellular matrix molecule comprises microcarriers encapsulated within the bioartificial organ.

4. The method according to claim 1, wherein the treatment further comprises adding growth factors or chemical compounds to the extracellular matrix molecules.

5. A bioartificial organ comprising:

(a) a biocompatible jacket;

(b) a core of living cells; and

(c) at least one growth factor within the bioartificial organ that is exposed to a treatment that inhibits cell proliferation or promotes cell differentiation comprising coating the growth surface with at least one extracellular matrix molecule,

wherein the treatment comprises coating the growth surface with an effective amount of at least one extracellular matrix molecule, the extracellular matrix molecule coating formed by culturing extracellular matrix molecule-forming cells on the growth surface and then lysing the extracellular matrix molecule-forming cells to produce a coating of at least one acellular extracellular matrix molecule on the growth surface.

6. The bioartificial organ of claim 5, wherein the growth surface is selected from the group consisting of a luminal surface of the bioartificial organ, an inner scaffold surface, an inner support surface, an internal membrane, and a surface of a microcarrier encapsulated within the bioartificial organ.

7. The bioartificial organ of claim 5, wherein growth factors or chemical compounds are added to the extracellular matrix molecule.

* * * * *

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Modulation of Extracellular Matrix by Angiotensin II: Stimulated Glycoconjugate Synthesis and Growth in Vascular Smooth Muscle Cells

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Summary: A role for angiotensin II (Ang II) in the pathogenesis of hypertension and atherosclerosis was studied using cultured vascular smooth muscle cells from spontaneously hypertensive rats. Chronic exposure of vascular smooth muscle cells, cultured in the presence of 1% plasma-derived serum, to Ang II resulted in a dose-dependent stimulation in growth and incorporation of radiolabeled matrix precursors into extracellular matrix–associated glycoconjugate material. The hormone also stimulated the incorporation of [^{3}H]glycine into extracellular matrix glycoproteins and proteoglycans synthesized by cultures rendered quiescent by maintenance on serum-free medium for 48 h prior to exposure to Ang II. This was negated in the presence of saralasin. In quiescent cultures, a single exposure to angiotensin induced a rapid induction of mRNA coding for the extracellular matrix glycoprotein thrombospondin. Similar results were obtained with cells maintained on medium containing 1% plasma-derived serum; however, the levels of induction were reduced by this procedure. This study demonstrated that Ang II was capable of stimulating both growth and matrix elaboration by cultured vascular smooth muscle cells. These observations are indicative of a pathophysiological role for the vasoconstrictor peptide, which may contribute significantly to the development of hypertension. **Key Words:** Angiotensin—Growth—Matrix elaboration.

Vascular smooth muscle cells (VSMCs) retain their ability to undergo reversible "dedifferentiation" from the contractile to the proliferative/secretory phenotype, a process that has been termed "phenotypic modulation" (1). This unique property of VSMCs may partly explain their involvement in the vascular pathologies of hypertension and atherosclerosis, which in the latter case leads to blood vessel occlusion and mortality (2–4). The stimulus for this phenotypic modulation was presumed to arise as a consequence of damage to the endothelium and the exposure of the medial VSMCs to growth factors such as platelet-derived growth factor (PDGF) and other blood cell–associated mitogens (5,6). It is now clear, however, that endothelial desquamation is not required a priori for neointimal formation, which arises as a consequence of some of the medial VSMCs undergoing conversion to the proliferative phenotype (7–10). Therefore, it is possible that normal components of the intact blood vessel wall, either alone or in combination, may be capable of initiating the "modulation" process in VSMCs. Nonetheless, regardless of how the phenotypic conversion occurs (as a consequence of endothelial damage or from other causes), it is the maintenance of the modulated VSMCs in their proliferative form for a prolonged period that leads to extensive myointimal thickening and vessel occlusion. An important component of vascular tissue that may play a key role in this pathologically serious process is the extracellular matrix (ECM). The latter, once considered to be no more than an inert scaffold for cell attachment, has now been demonstrated both to regulate and even possibly initiate phenotypic expression (11–15). A number of individual components of the ECM have been shown to be capable of stimulating intracellular events via interactions though their specific cell-surface integrin receptors (16,17).

Some recent reports in the literature have suggested that angiotensin II (Ang II) may play an important pathophysiological role in hypertension and

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atherosclerosis (18–22). The vasoconstrictor peptide elicited the induction of nuclear proto-oncogenes and protein synthesis (including that of growth factors) in quiescent VSMCs, both of which may subsequently contribute to myointimal thickening (18). Furthermore, in some tissues, there is evidence for a local production of Ang II that is independent of the plasma renin–angiotensin system (23,24). Within such tissues, a limited, local production of Ang II may be an important stimulus for extracellular matrix synthesis and remodeling, characteristic of the vessel wall structural changes in hypertension. Recently, it has been shown that neointimal formation as a consequence of endothelial denudation by balloon catheterization in rat carotid arteries could be inhibited by 80% in animals maintained on diets containing inhibitors of angiotensin-converting enzyme (25). Such findings strongly suggest that Ang II may indeed play a pathological role in the progression of vascular disease and that this effect may be mediated via its influence upon structural elements (extracellular matrix) of the vessel wall. We have addressed some of these questions in experiments with VSMCs isolated from spontaneously hypertensive rats (SHRs) and report on our findings here.

METHODS

Cell culture

The procedures employed for the isolation, characterization, and propagation of vascular smooth muscle cells from spontaneously hypertensive rats (SHRs) have all been described in detail elsewhere (26). All tissue culture chemicals and supplies were obtained from Gibco AG (Basel, Switzerland). Reagents and antibodies used for immunocytochemical characterization of cells were purchased from Dakopatts, IG-Instrumenten Gesellschaft (Zurich, Switzerland). Cultures were normally maintained in medium containing 10% fetal calf serum (FCS). Prior to experimentation, VSMCs were rendered quiescent by 48 h of serum deprivation (26,27). Extracellular matrices elaborated by cultured VSMCs were prepared and analyzed by enzymatic digestion as described previously (28) using trypsin (EC 3.4.21.4) supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). For these experiments, cells were cultured in the continuous presence of Na ascorbate and with either [^{3}H]glycine (19 Ci/mmol); [^{3}H]glucosamine (20 Ci/mmol); [^{3}H]fucose (40 Ci/mmol); or [^{35}S]Na_2SO_4 (369 Ci/mmol) all obtained from Amersham (Zurich, Switzerland), for the final 24 h prior to matrix isolation, in order to radiolabel extracellular matrix proteins as described previously (28).

Preparation of plasma-derived serum

Plasma-derived serum (PDS) was prepared essentially as described by Ross and Kariya (29) from bovine citrated blood. Platelet-depleted plasma was incubated at 37°C for 2 h in the presence of 20 m*M* $CaCl_2$ and the resulting fibrin clot broken up and removed by centrifugation. Following exhaustive dialysis against 100 m*M* Tris-HCl, pH 7.4, serum was passed over a carboxymethyl (CM)–cellulose column and protein eluted with dialysis buffer. PDS was sterilized by filtration. Batches of PDS were tested for their ability to stimulate the incorporation of [^{3}H]thymidine (Tdr) into DNA as described previously (27) when added to serum-free medium (final concentration of 1%) and in comparison with 10% FCS. Samples that stimulated [^{3}H]Tdr incorporation to levels significantly higher that serum-free medium alone (± 8% of the level of incorporation of 10% FCS) were rejected.

Preparation of RNA for Northern analysis

Following stimulation in serum-free (SF) or 1% PDS medium, cell layers were rinsed with PBS and cells lysed with guanidine isothiocyanate buffer (GT buffer). Total cellular RNA was prepared by standard CsCl gradient methodology and Northern blotted onto nylon filters (Hybond-N, Amersham) following electrophoresis through 1.2% agarose gels containing 2.2 *M* formaldehyde as described (27). Blots were hybridized to random primed cDNA probes labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) supplied by Amersham according to standard procedures already described (27). The cDNA probe used in these studies was a 1.3 kilobase cDNA fragment (clone λTS33) for thrombospondin (TSP) generously provided by Dr. P. Bornstein (30). After hybridization, blots were washed at high stringency using standard saline citrate buffer (SSC) and 0.1% SDS in SSC (SSC/SDS) in the following sequential protocol: 2 × SSC at room temperature; 2 × SSC/SDS; 1 × SSC/SDS; and 0.1 × SSC/SDS (all at 65°C); washes were for 20 min in all cases. All blots were rehybridized after stripping (10 min at 100°C in 10 m*M* Tris-HCl), with a random primed 1.5 kilobase cDNA probe (clone pMF 48) for MHC class I antigen (31) to facilitate the normalization of data in regard to the small variabilities in the amounts of RNA present. Blots were checked after stripping by overnight autoradiography using Kodak X-Omat film. Exposure times for hybridized blots using a single intensifying screen were 18–20 h for all probes except MHC cDNA, where 6–8 h was used routinely.

Statistical analysis

Data are expressed as the means ± SD unless stated otherwise and Student's *t* test for paired or unpaired data (whichever was relevant) used as the basis for the assessment of levels of significance.

RESULTS

When VSMCs were maintained for 12 days in the presence of medium containing 1% PDS, they exhibited a flat polygonal morphology, as viewed by phase-contrast light microscopy, which was atypical for modulated smooth muscle cells cultured in the presence of various levels of FCS (1). In contrast, cells maintained on the same medium with daily additions of Ang II (10^{-7} *M*) exhibited a typical morphological appearance of passaged smooth muscle cells maintained in the presence of 10% FCS. These morphological changes were only apparent after 6–8 days in culture and could be correlated with the onset of full extracellular matrix production (27). This chronic exposure of cells to Ang II in 1% PDS medium also led to a dose-de-

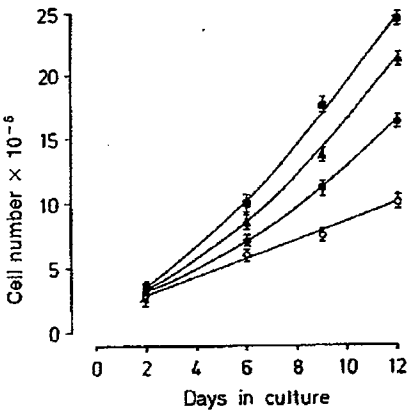

FIG. 1. Growth kinetics of vascular smooth muscle cells in the presence and absence of Ang II. Cells (VSMCs) were plated (10^5 cells/well) into 12-well multiwell plates in normal media containing 10% FCS, and when cell attachment was complete (~12 h), this was exchanged for media containing 1% plasma-derived serum (PDS) in place of FCS. The following day (24 h after plating), medium was again exchanged with PDS media containing either Ang II (10^{-7} *M*, ■–■; 10^{-8} *M*, ▲–▲; 10^{-9} *M*, •–•) or phosphate-buffered saline (PBS; open symbols). Medium was replaced routinely every 2 days and cell numbers were determined at the times shown. The data represent means ± SD of the values from experiments (n = 3) performed on different isolates of VSMCs.

pendent increase in cell number when determined over a full 12-day growth period (Fig. 1). At 12 days after plating, the number of cells in wells exposed to 10^{-7} *M* Ang II was 2.5×10^6 cells/well, as compared to 10^6 cells/well for control (unsupplemented cultures).

When VSMCs were maintained in the absence or presence of Ang II for 12 days and their synthesis of extracellular matrix glycoconjugates, there was a dose-dependent stimulation in the incorporation of all of the radioactive matrix precursor molecules tested (Fig. 2). The incorporation of [^{3}H]glycine into matrix material was stimulated approximately five-fold at the highest dose of angiotensin used (10^{-7} *M*), and [^{3}H]glucosamine incorporation into matrix proteoglycans and glycoproteins increased by more than 10-fold, which indicated a possible elevation in glycosylation of these molecules (Fig. 2A). When the incorporation of [^{3}H]fucose and [^{35}S]Na_2SO_4 was determined under the same conditions, it was apparent that there was a dose-dependent stimulation in the synthesis of both proteoglycans ([^{35}S]) and glycopeptides ([^{3}H]; Fig. 2B).

Quiescent VSMCs also showed an increased stimulation of radioactive precursor molecules into extracellular matrix-associated glycoconjugates when exposed to Ang II (10^{-7} *M*) for periods up to 24 h (Fig. 3). The data are indicative of a stimulation in the synthesis of both classes of glycoconjugates (proteoglycans and glycoproteins); the relative increase in the incorporation of [^{3}H]glucosamine as compared to [^{3}H]glycine was again apparent when cells were stimulated with Ang II under serum-free conditions. When quiescent cells were stimulated with the vasoconstrictor peptide in the presence of an equal concentration of saralasin (both 10^{-7} *M*), the stimulated incorporation of [^{3}H]glycine was negated (Fig. 4). Over prolonged periods of stimulation (> 20 h), the effect of the angiotensin II antagonist was no longer observed, which is consistent with its known instability in solution (data not shown).

Ang II, in the absence of additional growth factors or hormones, induced a rapid stimulation (approximately 10-fold by 3 h) of TSP mRNA expression in quiescent VSMCs from SHRs (Fig. 5A). When similar experiments were performed on cultures maintained on medium containing 1% PDS, the levels of

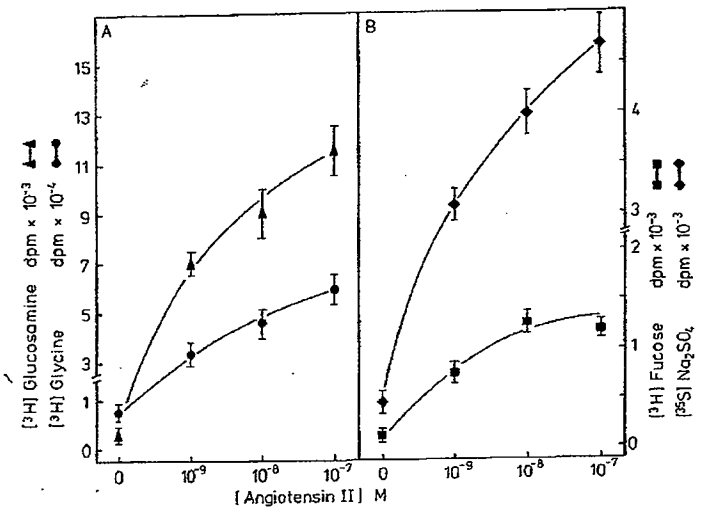

FIG. 2. Dose-dependent incorporation of radiolabeled isotopic precursors into extracellular matrix glycoconjugate material by vascular smooth muscle cells. Cells were plated into 12 well multiwell plates in normal medium containing FCS and this was exchanged for PDS media with or without the presence of angiotensin (Ang II) as described for Fig. 1 and in the Methods section. Routine medium changes were performed and sodium ascorbate (ASC; 50 μg/ml final concentration) was added daily. After 11 days, medium was replaced with medium containing the individual radioisotopic precursors as shown in the figure. Matrix material was isolated 24 h later. Cell-free matrix material was digested with trypsin to assess the levels of incorporation of the radiolabeled precursors into matrix glycoconjugates as described (27). Determinations were performed in quadruplicate in experiments (n = 4) performed with different cell isolates. The data represent the means ± SD of the values (dpm) obtained in one typical experiment.

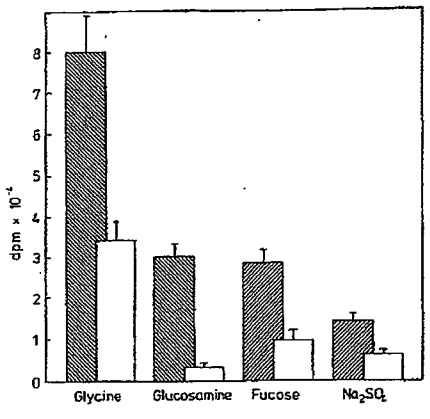

FIG. 3. Incorporation of radiolabeled isotopic precursors into extracellular matrix material by quiescent smooth muscle cells. Cells were plated (10^6 cells/well) into 12 well multiwell plates as described (in Fig. 1), except that after 24 h medium was exchanged for serum-free medium (containing sodium ascorbate) and cells maintained for a further 48 h. Quiescent cells were then treated with fresh SF medium in the presence (▨) or absence (□) of Ang II (10^{-7} *M*) and with the addition of the radioisotopes indicated in the figure. Matrix material was isolated and analyzed. The data represent the means ± SD of the values (dpm) obtained in experiments (n = 3) performed using different VSMC isolates.

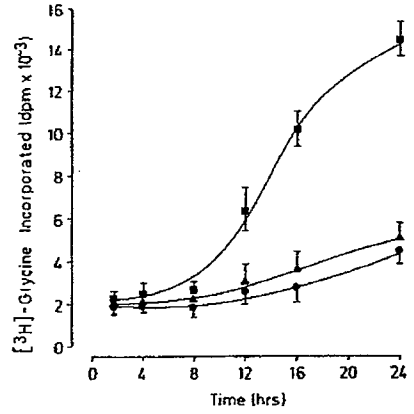

FIG. 4. Kinetics of incorporation of [^{3}H]glycine into extracellular matrix-associated glycoconjugate material by VSMCs. Cells (VSMCs) were plated into 12 well multiwell plates and rendered quiescent as described in Fig. 3. Serum-free medium containing [^{3}H]glycine (1 μCi/ml), sodium ascorbate, and either Ang II (■–■), Ang II + saralasin (▲–▲), or phosphate-buffered saline (●–●) was used to replace media on quiescent cells, which were then maintained for the times shown in the figure prior to isolation of cell-free matrix material. Angiotensin and saralasin were used at 10^{-7} *M* and experiments (n = 3) were performed using six wells/determination. Analysis and quantitation of data were carried out as for the other matrix experiments and the values shown represent the means ± SD.

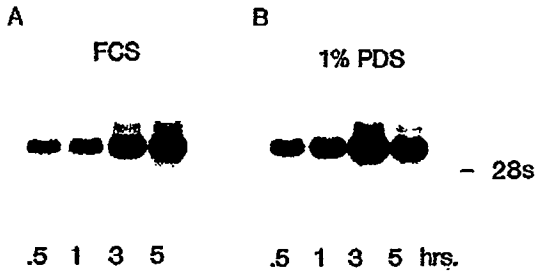

FIG. 5. Northern analysis of Ang II–induced thrombospondin expression in cultured vascular smooth muscle cells. Cells (VSMCs) plated into 150 mm culture dishes (5×10^6 cells/dish) were cultured either in the presence of media containing 10% FCS or 1% PDS until confluent and then medium was exchanged for either **(A)** SF or **(B)** 1% PDS medium for 48 h. Following incubation, cultures were exposed to Ang II (10^{-7} *M*) for the times indicated in the figure. Total cellular RNA was isolated, purified, and Northern analysis (using 20 μg of RNA/lane) was performed. The developed autoradiograph shown is typical for experiments (n = 3) performed using between 5×10^8 to 10^9 cells/plate for the extraction of total cellular RNA.

TSP transcripts induced by Ang II were reduced (approximately fivefold) and the kinetics of stimulation modified (Fig. 5B).

DISCUSSION

The morphological appearance of cultures maintained in the presence of 1% PDS and angiotensin II was typical of VSMCs grown in the presence of 10% FCS, namely the formation of "hills and valleys" with elongated cells that formed multilayers (1). This was not observed in the absence of angiotensin, which clearly suggested that the peptide exerted a mitogenic effect upon the supplemented cultures leading to the latter's distinct morphological appearance. This was confirmed when experiments were performed to assess the growth kinetics of VSMCs in the presence and absence of angiotensin. Growth stimulation of VSMCs by Ang II was only observed in the presence of medium containing 1% PDS and not under serum-free (SF) media conditions. The former media also exhibited a mild stimulatory effect on cell proliferation (Fig. 1) over the prolonged experimental period. This was not observed when cells from normotensive Wistar–Kyoto (WKY) rats were used in analogous studies to those reported here (data not shown). The explanation for this apparent specific stimulation in proliferation of VSMCs from SHRs by 1% PDS relates to the ability of VSMCs from SHRs to secrete and respond to the A chain homodimeric form of PDGF. VSMCs from SHRs and WKY rats appear to be capable of producing significant levels of the mitogen in culture, but only those from the hypertensive animals possess specific cell-surface receptors for $PDGF_{AA}$ (32).

The judicious use of a number of different isotopic precursors for extracellular matrix components indicated that Ang II stimulated the synthesis of matrix-associated glycopeptides and proteoglycans by cultured VSMCs, regardless of whether they were maintained in either SF or 1% PDS media. [^{3}H]fucose is specifically incorporated into glycopeptides, whereas [^{35}S]Na_2SO_4 labels only sulfated glycosaminoglycan chains on proteoglycan core proteins.

Northern analysis indicated that one candidate for the increased synthesis of glycopeptide material by cells exposed to Ang II was TSP (Fig. 5). Although chronic exposure of VSMCs to 1% PDS reduced their responsiveness to angiotensin with respect to TSP gene transcription, there was still a significant enhancement in the levels of TSP transcripts in treated cells (Fig. 5B). Since it has been demonstrated that PDGF specifically stimulated TSP gene expression (33) and also since rat VSMCs secrete this compound into conditioned media on stimulation with Ang II (20), we initially presumed that the mechanism of TSP induction by the vasoconstrictor peptide was related to its ability to stimulate PDGF production. However, the kinetics of PDGF production are not consistent with such a mechanism, since TSP induction occurred more rapidly than that of PDGF (20). Therefore, it is possible that Ang II is capable of inducing TSP expression in its own right, possibly via the putative AP-1 site(s) present on the TSP genome (30).

A pathophysiological role for TSP in vascular tissue has been suggested by its increased presence in the extracellular matrix associated with atherosclerotic lesions and its mitogenic effects on VSMCs in combination with epidermal growth factor (33–35). In culture, it has been shown to be essential for the maintenance of growth of VSMCs (36). This has also been correlated with the ability of heparin to inhibit growth of VSMCs and to prevent the interaction of TSP with its specific cell-surface integrin receptors (37). The rapid, transient induction of TSP expression by PDGF is analogous to its effect on the nuclear protooncogenes c-fos, c-myc, and c-jun and has resulted in the TSP genes becoming classified with the family referred to as "early response genes" (33,36). It has been suggested that induction of TSP expression in cultured VSMCs is specific to PDGF. However, although a number of typical growth factors were tested with respect to their ability to stimulate TSP expression, none of the vasoactive peptide hormones such as angiotensin, vasopressin, and endothelin were studied (34).

The relevance of our findings with regard to Ang II influence on the synthesis of extracellular matrix macromolecules by VSMCs relate to the ability of such compounds to elicit responses in the vascular cells. Turnover of extracellular matrix material is a relatively slow process and therefore any effects that such material may have upon the cells that exist within the matrix mileu may be prolonged. It remains to be clearly demonstrated that in vascular tissue an altered extracellular matrix can exert an effect on the cells it surrounds; however, there is an accumulation of evidence to suggest that, at least in culture, this does occur (11–17). This may therefore be an explanation of the apparent irreversibility of the structural changes that occur as a consequence of elevated blood pressure, even under conditions where this can be significantly reduced by therapeutic means.

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(54) Title: CONJUGATES OF GROWTH FACTOR AND BONE RESORPTION INHIBITOR

(57) Abstract

A method for stimulating bone formation in an animal includes the step of administering to the animal an effective amount of a conjugate of a growth factor and a hydrophilic polymer. Also, a composition for treating osteopenic bone disease includes a conjugate of a growth factor and a hydrophilic polymer. Preferred conjugates include, for example, a TGF-β as the growth factor, and a polyethylene glycol as the hydrophilic polymer. Hydrophilic polymer-conjugated growth factors according to the invention can stimulate bone formation at lower dose levels at which the growth factor, unmodified, is ineffective; and hydrophilic polymer-conjugated growth factors according to the invention promote a net increase in bone formation at higher dose levels at which the growth factor, unmodified, causes a net reduction in bone mass, owing to stimulation by the growth factor of bone resorption together with bone formation. An inhibitor of bone resorption is optionally added to decrease bone resorption and thus enhance bone formation.

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CONJUGATES OF GROWTH FACTOR AND BONE RESORPTION INHIBITOR

Cross-Reference to Related Applications

This is a continuation-in-part of pending United States Patent Application Serial No. 08/051,508, filed April 22, 1993.

Background of the Invention

Technical Field

This invention relates to the use of transforming growth factors in treatment of systemic disease, and, particularly, to the use of molecules in any of the "TGF-β Superfamily" of proteins, such as the beta-type transforming growth factors ("TGF-βs"), the bone morphogenetic proteins ("BMPs"), the activins, and the inhibins, for promoting bone formation *in vivo*, particularly for treatment of osteopenic bone diseases.

Background Art

Various of the so-called growth factors can be administered systemically for therapeutic effect. Many such growth factors produce harmful or undesirable systemic effects when administered in quantities sufficient to produce the desired therapeutic effect. It is generally recognized that harmful or undesirable effects of therapeutic agents can be reduced by targeted delivery of the agent, by which a relatively smaller quantity of the agent administered to the subject localizes at the targeted

site in a relatively higher concentration. In one approach to targeted delivery of an agent, a targeting molecule having an affinity for the targeted tissue, such as a monoclonal antibody that binds cell surface receptors of targeted cell types, is conjugated to a molecule of the agent. Administered systemically, the antibody-agent conjugate circulates until it reaches a cell of the target type, whereupon the antibody binds the cell surface receptor, localizing the antibody-agent at the targeted site.

The family of peptides known as TGF-β can regulate both cell growth and cell differentiation. Depending upon the particular cell type, the peptides of the TGF-β family can stimulate or inhibit cell proliferation. Almost all tissues from all species of animals which have been examined contain TGF-βs of some type.

Copending U.S. Patent Application Serial No. 07/698,467 describes compositions for treating bone loss that include a bone growth factor, such as TGF-β, activin, or bone morphogenic protein ("BMP"), chemically conjugated (preferably via a crosslinker) to a targeting molecule having an affinity for bone, such as tetracycline, calcein, bisphosphonate, polyaspartic acid, polyglutamic acid, aminophosphosugars, or estrogen. Preferred crosslinkers, according to the '467 application, include PEG having average molecular weights between about 200 and about 10,000 daltons.

Several examples of references in the art to modifying proteins by conjugation to polymers, to alter the solubility, antigenicity, and physiological clearance of the protein may be cited. U.S. Patent No. 4,179,337 describes coupling polypeptides such as enzymes and insulin to polyethylene glycol ("PEG") or polypropylene glycol ("PPG") of 500 to 20,000 daltons molecular weight to provide physiologically active

water soluble compositions having reduced immunogenicity; U.S. Patent No. 4,261,973 describes reducing the immunogenicity of several proteins by conjugating the proteins with polyethylene glycol ("PEG") or polypropylene glycol ("PPG"). U.S. Patent No. 4,301,144 describes increasing the oxygen carrying capacity of hemoglobin by conjugating the hemoglobin with PEG and other polymers. EP 0 98,110 describes increasing the half-life of an enzyme or an interferon by coupling with a polyoxyethylene-polyoxypropylene ("POE-POP") block polymer. Abuchowski *et al.* (1984), *Cancer Biochem. Biophys.*, 7:175-86, describes reduced immunogenicity and increased half-lives in serum of a variety of enzymes conjugated with PEG. Davis *et al.* (1981), *Lancet*, 2:281-83, describes modifying the enzyme uricase by conjugating with PEG to provide uric acid metabolism in serum having a long halflife and low immunogenicity; Nishida *et al.* (1984), *J. Pharm. Pharmacol.*, 36:354-55, describes orally administering PEG-uricase conjugates to chickens, demonstrating decreased serum uric acid. Inada *et al.* (1984), *Biochem. & Biophys. Res. Comm.*, 122:845-50, describes rendering lipoprotein lipase soluble in organic solvents by conjugation with PEG; Takahashi *et al.* (1984), *Biochem. & Biophys. Res. Comm.*, 121:261-65, describes conjugating horseradish peroxidase (HRP) with PEG to render the enzyme soluble in benzene. Abuchovski *et al.* (1977), *J. Biol. Chem.*, 252(11):3578 ff., describes conjugating bovine serum albumin with PEG resulting in reduced immunogenicity and extended circulating life in the blood; and Abuchovski *et al.* (1977), *J. Biol. Chem.*, 252(11):3582 ff. describes conjugating bovine liver catalase with PEG resulting in reduced immunogenicity and enhanced half lives in the blood, and substantially retained enzymatic activity.

Summary of the Invention

We have discovered that a conjugate of a recombinant TGF-β and a hydrophilic polymer such as a polyethylene glycol ("PEG"), administered to an animal *in vivo*, can be substantially more effective for stimulating bone formation than unmodified TGF-β; and, in particular, a PEG-TGF-β conjugate can be effective for stimulating bone formation *in vivo* when administered at dose levels at which unmodified TGF-β alone is ineffective. Moreover, substantially less bone resorption results from PEG-TGF-β conjugate administration *in vivo* than from administration of unmodified TGF-β at dose levels at which unmodified TGF-β is effective in stimulating bone formation *in vivo*.

The present invention also offers *in vivo* combination therapy for stimulating new bone formation through the combined administration of the PEG-TGF-β and an agent which inhibits bone resorption.

Disclosure of Invention

In one aspect, in general, the invention features a method for treating a systemic disease condition by administering to the animal an effective amount of a conjugate of a growth factor and a hydrophilic polymer. In particular embodiments, the growth factor is a bone growth factor and the systemic disease condition is treated by stimulating bone formation.

A "bone growth factor", as that term is used herein, includes any of the TGF-β family of growth factors, and includes activin and bone morphogenetic proteins ("BMP").

In preferred embodiments the bone growth factor is a TGF-β, such as TGF-β2, and more preferably is a recombinant TGF-β, such as recombinant TGF-β2. "TGF-β", as that term is used herein, includes TGF-β1,

TGF-β2, TGF-β3, TGF-β4 and TGF-β5, and heterodimers thereof; and, more broadly, "TGF-β" means and includes any molecule that competes with binding of the native form of TGF-β for any of the cell surface TGF-β-binding proteins, including any of the TGF-β receptors types I through IX that have to date been characterized.

A "hydrophilic polymer", as that term is used herein, is a synthetic or natural polymer having an average molecular weight and composition that render the polymer essentially water soluble. Most hydrophilic polymers have this property by virtue of their having a sufficient number of oxygen atoms (less frequently nitrogen atoms) available for forming hydrogen bonds in aqueous solution. Hydrophilic polymers generally used herein include PPG, PEG, POE, polytrimethylene glycols, polylactic acid, and derivatives thereof. Particularly suitable hydrophilic polymers include a polyethylene glycol $-(CH_2CH_2O)_n-$ ("PEG"), a polypropylene glycol $-(CH_2CH_2CH_2O)_n-$ ("PPG"), or a hydrophilic carbohydrate or polysaccharide; preferred hydrophilic polymers include, for example, PEGs or PPGs having a molecular weight between 200 and 100,000, such as, for example, PEG 5000, PEG 1700, or PEG 35,000. Preferably, where the hydrophilic polymer is a PEG and the growth factor is a TGF-β2, 1 - 14 molecules of the hydrophilic polymer, and more preferably 1 - 7 molecules of the hydrophilic polymer, are attached to each TGF-β2 homodimer; that is, the PEG is present with TGF-β2 homodimer in a molar ratio preferably in the range about 1:1 to about 14:1, and more preferably in the range about 1:1 to about 7:1. Other suitable polymers include POE-POP block polymers and copolymers.

As used herein, a "conjugate" of a hydrophilic polymer and a growth factor is a

composition in which the hydrophilic polymer is attached to the growth factor *via* a covalent bond.

As used herein, the term "inhibition of bone resorption" refers to prevention of bone loss, especially the inhibition of removal of existing bone either from the mineral phase and/or the organic matrix phase, through direct or indirect inhibition of osteoclast formation or activity. Thus, inhibitor of bone resorption refers to agents that prevent bone loss by the direct or indirect inhibition of osteoclast formation or activity.

In another general aspect, the invention features a composition for stimulating bone deposition, including a conjugate of a growth factor and a hydrophilic polymer.

In still another aspect, the invention features a composition for stimulating bone deposition, including a conjugate of a growth factor and a hydrophilic polymer and an inhibitor of bone resorption.

While we do not wish to be bound to any particular theory of operation of the hydrophilic polymer — growth factor conjugates according to the invention, we offer the following observations.

The growth factor may be brought into more effective proximity to the tissue site on which the growth factor is effective, when conjugated with a hydrophilic polymer according to the invention. This may result from a more or less specific affinity of the hydrophilic polymer (or of the conjugate) for the tissue. PEG-TGF-β2 conjugates according to the invention may effectively localize to bone following systemic administration. As a result, the action of TGF-β2 on both osteoblasts and osteoclasts may be enhanced or prolonged; and an *in vivo* inhibition of osteoclast activity by TGF-β2 (as can be observed *in vitro*) can be effected, resulting in a net increase in

bone. For stimulation of bone formation, therefore, preferred hydrophilic polymers may (either in and of themselves or when conjugated with a bone growth factor) have a specific affinity for bone tissue.

Alternatively, the pharmacokinetics of the growth factor may be altered by conjugation with a hydrophilic polymer according to the invention. For example, in the bone formation stimulation example, a differential effect of the bone growth factor (such as TGF-β2) on osteoblasts (as compared with osteoclasts) may result from conjugation with the hydrophilic polymer (such as PEG), resulting in a net increase in bone. Osteoblasts and osteoclasts may have different receptors for TGF-β, for example, binding different portions of a particular TGF-β molecule or binding with a greater or lesser affinity; and such differences may result in a differential effect of the hydrophilic polymer-conjugated growth factor on these two cell types.

We have further discovered that reduced toxic effects result from administration of a conjugate of a higher molecular weight PEG (for example, PEG 35,000) and a TGF-β2 at lower molar ratios, or lower degrees of PEG conjugation, that is fewer moles of PEG covalently attached to TGF-β (for example, in the range about 1:1 to about 3:1), at TGF-β2 dose levels effective for stimulating bone formation.

In another general aspect, therefore, the invention features a method for stimulating bone formation in an animal by administering to the animal a PEG-TGF-β2 conjugate in which the PEG has a molecular weight between about 5 kd and about 100 kd, and preferably about 35 kd, and in which the higher molecular weight PEG is present with the TGF-β2 homodimer in a molar ratio in the range about 1:1 to about 3:1.

As will be appreciated, the preferred molar ratio for a given combination of hydrophilic polymer and growth factor depends among other variables upon the molecular size of the hydrophilic polymer. In general, lower molar ratios of a larger than of a smaller hydrophilic polymer can be effective in a conjugate with a given growth factor. The mechanism of systemic clearance is different for circulating molecules of different sizes. The clearance mechanism for a particular growth factor-hydrophilic polymer conjugate depends among other factors upon the molecular size of the whole conjugate (*see, e.g.*, M.J. Knauf *et al.* (1988), *J. Biol. Chem.*, 263(28): 15064-70???. TGF-βs, for example, have a molecular size (dimer) about 26,000; preferred conjugates of hydrophilic polymers with TGF-βs have a molecular size at least about 55,000 or 60,000; such molecular sizes can be obtained by conjugation of PEG 5000 and a TGF-β at molar ratios at least about 6:1, or by conjugation of PEG 35,000 and a TGF-β at a molar ratio of at least about 1:1. Apparently, as the examples below demonstrate, a lower molar ratio of higher molecular size PEGs in PEG-TGF-β conjugates can be more effective than a higher molar ratio of lower molecular size PEGs. For conjugated TGF-β2, for example, the activity is significantly reduced or lost at molar ratios of PEG 5000 to TGF-β2 greater than 7:1.

The hydrophilic polymer — growth factor conjugate can be administered in conjunction with a bone resorption inhibitor. In preferred embodiments the conjugate is administered in conjunction with a bone resorption inhibitor such as, for example, an estrogen, a bisphosphonate or a calcitonin. Administration of the conjugate can commence either prior to, at the same time as, or following administration of the bone resorption inhibitor. The conjugate and bone resorption inhibitor can be

administered at least partly concurrently. Where administration is to be simultaneous, the conjugate and the bone resorption inhibitor may or may not be combined in a single composition.

Drugs which prevent bone loss and/or add back lost bone are often first tested in the ovariectomized rat. This animal model is well established in the art (see, for example, Wronski, et al. (1985) Calcif. Tissue Int. 37:324-328; Kimmel, et al. (1990) Calcif. Tissue Int. 46:101-110; and Durbridge, et al. (1990) Calcif. Tissue Int. 47:383-387; these references are hereby incorporated in their entirety). Wronski, et al. ((1985) Calcif. Tissue Int. 43:179-183)) describe the association of bone loss and bone turnover in the ovariectomized rat.

Examples of inhibitors of bone resorption include estrogens such as estradiol, tamoxifen, bisphosphonates, calcitonins, or other small peptides or molecules that may inhibit bone resorption. (Turner, et al. (1987) J. Bone Mineral Res. 2:115-122; Wronski, et al. (1988) Endocrinology 128:681-686; and Wronski, et al. (1989) Endocrinology 125:810-816; Pfeilshifter, et al. (1987) Proc. Natl. Acad. Sci. U.S.A. 84:2024-2028; Turner, et al. (1988) Endocrinology 122:1146-1150). An example of a small peptide is echistatin, which includes the arginine-glycine-aspartate (RGD) sequence which is recognized by some cell surface adhesion receptors and apparently disrupts osteoclast interactions (Fisher et al. (1993) Endocrinology 132: 1411-13). Another example of a bone resorption factor is OPF or osteoclastpoietic factor (PCT Publication WO 93/01827 published 4 February 1993).

The entire molecule of a particular inhibitor may be used, or alternatively, only a functional part of the inhibitor molecule may be used.

Bisphosphonates include, but are not limited to, pamidronate, alendronate, residronate and tiludronate.

Description of Preferred Embodiments

There follow illustrative protocols for making hydrophilic polymer — growth factor conjugates according to the invention, showing by way of example protocols for production of PEG-TGF-β2 conjugates. Other hydrophilic polymers than polyethylene glycols, and other growth factors than TGF-βs can be coupled to make the conjugates of the invention. Adjustment of steps and particular parameters given in the protocols can be made as appropriate for the particular materials and according to the custom of the particular laboratory, all without undue experimentation and within the ordinary skill of the art.

Examples of chemistries for conjugating or coupling proteins to hydrophilic polymers have been summarized in the art. *See, e.g.*, Tyle *et al.*, Eds. (1990), *Targeted Therapeutic Systems*, Marcel Dekker, NY; and Means *et al.* (1990), *Bioconjugate Chemistry*, Vol. 1, pp. 2-12.

Modes of Carrying out the Invention

Construction of hydrophilic polymer — growth factor conjugates

Generally, the growth factor can be conjugated at a reactive group to the selected hydrophilic polymer or polymers. Reactive groups on the growth factor include, but are not limited to, carboxyl groups of the polypeptide C-terminus or of aspartic acid or glutamic acid residues, amino groups of the polypeptide N-terminus or of lysine residues, imidazole functions of histidine residues and phenolic functions of tyrosine residues, sulfhydryl groups of residues, and guanidine groups of arginine residues.

The molar ratio of hydrophilic polymer molecules to growth factor molecules can be controlled by selection of specific conjugation chemistries (for example, by reacting the polymer predominantly with primary amine substituents on lysine residues, of which there are a fixed number on any given growth factor molecule) and, as will be appreciated, by control of reaction conditions such as temperature or pH.

For example, the bone growth factor TGF-β contains a number of available amino, carboxyl, and hydroxy groups that may be used to bind the hydrophilic polymer. The hydrophilic polymer may be connected using a "linking group", as the native hydroxy or amino groups in TGF-β and in the hydrophilic polymer frequently require activation before they can be linked. Thus, for example, a compound such as a dicarboxylic anhydride (*e.g.*, glutaric or succinic anhydride) can be employed to form a polymer derivative (polymer glutarate or polymer succinate, *e.g.*, using PEG and succinic anhydride, a PEG-succinate), which can then be activated by esterification with a convenient leaving group (*e.g.*, N-hydroxysuccinimide, N,N'-disuccinimidyl oxalate, N,N'-disuccinimidyl carbonate, or the like). The activated polymer is then allowed to react with the bone growth factor, to form the hydrophilic polymer — bone growth factor conjugate.

Preferred dicarboxylic anhydrides for use in forming polymer-glutarate compositions include glutaric anhydride, adipic anhydride, 1,8-naphthalene dicarboxylic anhydride, and 1,4,5,8-naphthalenetetracarboxylic dianhydride. Suitable crosslinkers have chemistries well known in the art, and they are commercially available.

The resulting hydrophilic polymer — growth factor conjugate can be purified using a standard

technique, such as by reverse-phase high performance liquid chromatography ("RP-HPLC"), size-exclusion HPLC ("SEC-HPLC"; *e.g.*, tetrahydrogel-HPLC), or ion-exchange chromatography. RP-HPLC is preferably performed using a C18 column using gradient elution with 80 - 100% acetonitrile, ethanol or isopropanol with 0.1% trifluoroacetic acid ("TFA") as the eluting solvent. For SEC-HPLC, a preferred running buffer can be 5 mM sodium acetate at pH 5.5, preferably including an organic solvent, urea or a detergent.

PEG-TGF-β Conjugates

For example, a PEG-TGF-β conjugate can be made according to the following protocols, which make specific reference to formation of rTGF-β2(PEG 5000)$_6$ from PEG 5000 and rTGF-β2 and to formation of rTGF-β2(PEG 35,000)$_{1-3}$ from PEG 35,000 and rTGF-β2.

TGF-β is difficult to dissolve in solutions of appropriate pH for coupling to hydrophilic polymers. In a preferred protocol, therefore, the TGF-β is lyophilized in the absence of a carrier protein or is held in solution in acid/organic solvent. For use TGF-β is dissolved in a mild acid, preferably about 10 mM HCl, in the presence of a disaggregating reagent such as an organic solvent (*e.g.*, 40% acetonitrile, ethanol or propylene glycol; or a combination of organic solvents such as 30% ethanol, 15% propylene glycol). The solution is then neutralized by adding a base, preferably NaOH (1 N solution) in buffered saline (*e.g.*, phosphate buffered saline). The final solution preferably contains about 40 - 50% DMSO or CH_3CN to solubilize the TGF-β and to prevent aggregation, thus preserving TGF-β activity.

rhTGF-β2(PEG 5000)$_6$

In a preferred protocol, the activated PEG 5000 is a succinimydyl ester made, for example, as follows. Monomethylpolyethylene glycol, average

molecular weight about 5,000 daltons, ("mPEG 5000") is reacted with glutaric anhydride to form mPEG glutarate. The glutarate derivative is then reacted with N-hydroxysuccinimide to form a succinimydyl mPEG glutarate. The succinimydyl ester is then capable of reacting with free amino groups (lysine residues) on the TGF-β to form a TGF-β — PEG conjugate.

The following specific protocol was used for making rhTGF-β2(PEG 5000)$_6$ in a 5 mg batch from recombinant human TGF-β2 (rhTGF-β2) and methoxypolyethylene glycol succinimydyl succinate 5000 (M-S-PEG 5000). 5.2 mg/ml rhTGF-β2 (200 nmol rhTGF-β2) available from Genzyme Corp., Cambridge, MA, was combined with 5.5 ml acetonitrile, 1.5 ml HPLC-grade water, 1.4 ml 10x phosphate buffered saline (10x PBS) and 100 μl 0.1 N NaOH to make about 13.70 ml total volume, with a protein concentration of 0.38 mg/ml, 48% organic solvent, at pH 7.2. M-S-PEG 5000 (Sigma, lot 11H8040) (8 μmol; 40 mg), stored dry at -20 °C, was added (either directly or dissolved in acetonitrile) at a molar ratio of 40:1, to the rhTGF-β2 mixture, and permitted to react for 2 hours at room temperature. The resulting mixture was then diluted at least 1:3 with 0.1% TFA, the pH was adjusted to between pH 2 and pH 4 using 1% TFA, and the mixture was fractionated by C18-RP-HPLC (Vydac 218TP510, 1 x 25 cm). Solvent A was 0.1% TFA and solvent B was 90% acetonitrile in A. In a 1%/min. gradient of A to B, unreacted TGF-β2 eluted first at 41% solvent B followed by TGF-β with one PEG 5000 molecule attached. In a subsequent gradient at 0.5%/min. from 41-50% solvent B, TGF-β2 having increasingly higher amounts of PEG 500 eluted from the column. By stepping up solvent B to 62%, a PEG-TGF-β2 conjugate having a high molar ratio of PEG 5000 to TGF-β2 eluted. Fractions were further analyzed by SDS-PAGE under reduced and unreduced conditions.

TGF-β2 fractions having more than three PEG 5000 molecules attached were pooled for evaluation.

rhTGF-β2(PEG 35,000)$_{1-3}$

The following specific protocol was used for making rhTGF-β2(PEG 35,000)$_{1-3}$ from recombinant human TGF-β2 (rhTGF-β2) and bis-polyethylene glycol succinimydyl carbonate 35,000 (BSC-PEG 35,000). 2.6 mg/ml rhTGF-β2 in HCl, 20% EtOH, made according to the above-referenced production protocol, was combined with 2.0 ml acetonitrile, 1.0 ml propylene glycol (PG), 1.0 ml HPLC-grade water, 1.0 ml 10x PBS and 100 µl 0.1 N NaOH to make about 8.7 ml total volume. BSC-PEG 35,000, provided by Milton Harris, University of Alabama, Huntsville, AL, was added either (A) in a 5:1 mol ratio (20 mg) or (B) in a 2:1 mol ratio (8 mg), to the rhTGF-β2 mixture, to yield a protein concentration 0.268 mg/ml, 36.3% organic solvent, 10.3% PG, at pH 7.2. The mixture of rhTGF-β2 and BSC-PEG 35,000 was permitted to react for 90 min. at room temperature, pH 7.2. The resulting mixture was then diluted at least 1:3 with 0.1% TFA, the pH was adjusted to between pH 2 and pH 4 using 1% TFA, and the TGF-β2(PEG 35,000) was purified by C18-RP-HPLC according to the purification protocol referenced above. The resulting HPLC fractions were analyzed under non-reduced and reduced conditions on SDS-PAGE, 5-15% gradient. The protein concentration of pooled purified samples was determined at OD 280 nm, and aliquots were prepared for use as described above.

Pharmaceutical compositions containing hydrophilic polymer-growth factor conjugate

The PEG-TGF-β compositions of the invention are preferably administered by parenteral routes, intravenous injection, intranasal or bronchial aerosol, and the like. The compositions can be employed in sustained release vehicles, such as from a

slow-release carrier, or from a sustained release device that may be surgically implanted subdermally or within the peritoneal cavity.

Pharmaceutical formulations for administration of the hydrophilic polymer — bone growth factor according to the invention generally include an osteogenically effective amount of the bone growth factor in addition to a pharmaceutically acceptable excipient. Suitable excipients include most carriers approved for parenteral administration, including water, saline, Ringer's solution, Hank's solution, as well as solutions of lactose, dextrose, ethanol, glycerol, albumin, and the like. The compositions may optionally include stabilizers, antioxidants, antimicrobials, preservatives, buffering agents, surfactants, and other accessory additives, such as propylene glycol. A preferred mode of administration includes about 10-50% propylene glycol. The more preferred mode includes about 40% propylene glycol.

By way of example, saline or phosphate-buffered saline (PBS) can be a preferred vehicle for parenteral administration of rTGF-β—PEG. (Martin, *Remington's Pharmaceutical Sciences*, Mack Publ. Co., current edition, includes a discussion of suitable vehicles for parenteral administration; sections thereof relating to excipient vehicles and formulating are hereby incorporated by reference.)

The hydrophilic polymer — growth factor compositions of the invention may be formulated as solutions or suspensions, or they may be lyophilized for later reconstitution.

An "osteogenically effective amount" of hydrophilic polymer — growth factor conjugate, that is, an amount sufficient to effect treatment in the subject, will depend upon the particular growth factor and the particular hydrophilic polymer used in the

conjugate and the number of polymer molecules attached to each growth factor molecule in the conjugate, as well as the nature and severity of the condition to be treated, the age and general health of the subject, the specific activity of the composition, and other factors that may be determined by the practitioner of ordinary skill in the art of treating bone disease. Generally, doses of conjugate in the range 0.001 to 10 μg/kg body weight, and more preferably in the range 0.001 to 1 μg/kg body weight, and most preferably in the range 0.01 to 0.1 μg/kg body weight, should be effective. Hereinafter, weights or volumes given /kg refer to /kg of body weight.

Because the hydrophilic polymer — bone growth factor conjugates of the invention are substantially more effective for stimulating bone formation *in vivo* than the bone growth factor alone, effective systemic dosages of the conjugate according to the invention are much lower and may be less frequently administered than are effective dosages of the corresponding unconjugated growth factor. For stimulation of bone growth using PEG-TGF-β2 conjugates, for example, the number of molecules of the conjugate that need be administered systemically to achieve the desired treatment effect can be at least 10- to 100-fold lower than the number of molecules of unmodified TGF-β2 that must be administered to achieve the desired treatment effect. Thus, conjugate compositions according to the invention that have relatively lower activities as determined by an *in vitro* assay can be effective for treatment *in vivo*.

An effective dose for estrogen is about 1 μg/kg to about 1 mg/kg of body weight. An effective dose for bisphosphonates is quite variable but generally between about 0.05 μg/kg to about 15 mg/kg of body weight. An effective dose for calcitonin is

about 0.05 IU (International Units or Medical Research Council Units)/kg to about 2.5 IU/kg of body weight.

The combination of PEG-TGF-β and an inhibitor of bone resorption is useful for treating bone fractures, defects, and disorders which result in weakened bones such as osteoporosis (including postmenopausal, age-related and idiopathic), osteoarthritis, Paget's disease, osteohalisteresis, osteomalacia, bone loss resulting from multiple myeloma and other forms of cancer, and bone loss resulting from side effects of other medical treatment (such as steroids).

Industrial Applicability

The hydrophilic polymer — growth factor conjugates according to the invention can be administered for treatment of diseases where bone loss occurs, such as, for example, osteoporosis.

There follow examples illustrating effects on bone metabolism of administration of the hydrophilic polymer — bone growth factor conjugates of the invention, employing an animal model, as well as combination therapy of PEG-TGF-β with inhibitors or bone resorption.

Comparison of effects of rTGF-β2(PEG 5000)$_6$ and rTGF-β2 on bone formation in mice

In one example, groups of mice were treated by subcutaneous injection with two different doses of recombinant TGF-β2 ("rTGF-β2"), with two different doses of polyethylene glycol 5000 conjugated (6:1) with recombinant TGF-β2, or "rTGF-β2(PEG 5000)$_6$", or with a vehicle control. A variety of analyses were used to determine the effects of the treatments on bone formation.

More particularly, 10-week-old male C3H mice were divided into five treatment groups of six mice each. Mice in the first group were treated with mouse

serum albumin (MSA), a vehicle control; those in the second and third groups were treated with rTGF-β2 at doses of 20 μg and 0.1 μg per mouse (760 μg and 3.8 μg per kg body weight), respectively; and those in the fourth and fifth groups were treated with rTGF-β2(PEG 5000)$_6$ at doses of 0.1 μg and 0.02 μg per mouse (3.8 μg and 0.76 μg per kg body weight), respectively. (The body weight index is 26.4 g, the average weight of the animals at the start of the study.) Treatments were performed daily for eleven days by subcutaneous injections of 100 μl each in the tailbase. Demeclocycline was used as a fluorochrome label on day 1, and calcein was used on days 6 and 10 for histomorphometric analyses.

A variety of analyses were performed. Body weights of the animals were measured daily. At the end of the treatment period the animals were sacrificed, and analyses were performed as follows: hematology (white cell counts, red cell counts, packed cell volume (PCV), hemoglobin, platelet counts, differential blood cell counts); thymic cellularity; histology (liver, kidney, spleen); and bone histomorphometry (femur epiphysis).

The results can be summarized as follows. Mice treated with rTGF-β2(PEG 5000)$_6$ in daily doses of 3.8 μg/kg body weight showed increases in indices of bone formation in all measured parameters, with close to normal values for indices of bone resorption, resulting in an overall increase in cancellous bone mass (increases in percent trabecular area, trabecular thickness, trabecular number, decreases in trabecular separation). In contrast, bone formation in mice treated with unmodified rTGF-β in the same daily doses of 3.8 μg/kg body weight was not significantly different from bone formation in controls. Both bone formation and bone resorption indices were stimulated in mice treated with the higher dose of rTGF-β2, which

resulted in a net reduction of trabecular bone mass (decreases in percent trabecular area, trabecular thickness, trabecular number; increases in trabecular separation).

Comparison of effects of rTGF-β2(PEG 35,000)$_{1-3}$, rTGF-β2(PEG 5000)$_6$, and rTGF-β2 on bone

In another example, mice were treated by subcutaneous injection with rTGF-β2 (two different doses), with rTGF-β2(PEG 5000)$_8$ or rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ (two different doses for each molar ratio), with rTGF-β2(PEG 35,000)$_{1-3}$ (two different doses), or with a vehicle control. A variety of analyses were used to determine the effects of the treatments on bone cell morphology.

More particularly, 8 week-old-male C3H mice were divided into treatment groups. Mice in the first group were treated with MSA-PEG 5000, as a control; those in the second group were treated with 3 μg r-TGF-β2 admixed with PEG 5000 (uncomplexed); those in the third and fourth groups were treated with rTGF-β2 at doses of 15 μg and 3 μg per mouse, respectively; those in the fifth, sixth and seventh groups were treated with 3 μg per mouse rTGF-β2(PEG 5000) at molar ratios (PEG: rTGF-β2) of 8:1, 6:1, and 4:1, respectively; those in the ninth, tenth and eleventh groups were treated with 0.6 μg per mouse rTGF-β2(PEG 5000) at molar ratios 8:1, 6:1, and 4:1, respectively; and those in the eighth and twelfth groups were treated with rTGF-β2(PEG 35,000)$_{1-3}$ at doses of 3 μg and 0.6 μg per mouse, respectively. Treatments were performed once daily for four days by subcutaneous injections of 100 μl each in the tailbase.

A variety of analyses were performed. Body weights of the animals were measured daily. At the

end of the treatment period the animals were sacrificed, and analyses were performed as follows: hematology (white cell counts, red cell counts, packed cell volume (PCV), hemoglobin, platelet counts, differential blood cell counts); thymic cellularity; and qualitative femur histology.

The results can be summarized as follows. Very highly significant proliferation of osteoblast-like cells was observed in the femur slides of mice treated at 3 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$, as compared with controls. Such cell proliferation was interpreted as bone stimulation. Less dramatic but still highly significant proliferation of osteoblast-like cells was observed in femur slides obtained from mice treated at 0.6 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$. Significant proliferation of osteoblast-like cells was observed on the cortical and trabecular bone surfaces of the femur obtained from rTGF-β2(PEG 35,000)$_{1-3}$ treated mice. Generally, each dosage showed slightly greater effects on bone tissue than rTGF-β2(PEG 5000) at molar ratios 6:1 or 4:1.

Significant increases in RBC, hemoglobin and PCV, and significant decreases in platelets and thymic cellularity appeared in mice treated at 3 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$; significant decreases in bone marrow (femur) cellularity appeared in mice treated at 3 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$. Weight loss was high (about 20%) in mice treated at 3 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$.

Mice treated with rTGF-β2(PEG 5000)$_8$ gained weight slightly over the study period and showed no significant changes in hematologic, bone marrow or thymic analysis.

At the lower dosage (0.6 μg), while mice treated with rTGF-β2(PEG 5000)$_6$ showed significant increases in RBC, hemoglobin and % PCV, mice treated at 0.6 μg with rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$ showed no significant changes in hematologic or bone marrow or thymus analysis. Moreover, while weight loss was very high (about 9%) in mice treated with rTGF-β2(PEG 5000)$_6$ even at the lower dose, weight loss was only moderate (about 3.3%) in mice treated at 0.6 μg with rTGF-β2(PEG 5000)$_4$; and mice treated at 0.6 μg with rTGF-β2(PEG 35,000)$_{1-3}$ lost less than 1% of body weight over the study period and this treatment was about as effective in stimulating proliferation of osteoblast-like cells (which was interpreted as bone growth stimulation) as were the higher dosages of rTGF-β2(PEG 5000) at molar ratios 6:1 and 4:1.

Body weight losses, reduction in thymus cellularity and hematological changes are all toxic effects resulting from PEG-TGF-β treatment. In summary, coupling TGF-β with higher molecular size PEGs (for example, in the range about 10,000 to 100,000) at lower molar ratios (for example, in the range about 1:1 to 3:1) can produce PEG-TGF-β conjugates having greater effectiveness in bone stimulation at similar dosages and having similar effectiveness at lower dosages with substantially reduced toxic effects, than coupling TGF-β with lower molecular size PEGs (for example, in the range about 200 to 10,000) at higher molar ratios (for example, as high as about 8 PEG:1 TGF-β).

Comparison of effects of PEG-rTGF-β2 with and without bone resorption inhibitors on bone formation in rats

In one example, groups of ovariectomized (OVX) rats were treated by subcutaneous injections of PEG-rTGF-β2 with and without the bone resorption

inhibitor estradiol as a preliminary examination largely of safety.

More particularly, female rats were ovariectomized at 12 weeks of age and then lost bone until the start of the study when the rats were 95 weeks old. The OVX rats were given a dietary intake restricted to minimize the body weight gain that normally follows ovariectomy.

During the day prior to the study, OVX rats had their spinal bone mineral density (BMD) measured by DEXA. Based on spine BMD values, the OVX rats were then randomly assigned among the study groups described below, with reasonable attempts made to have mean BMD values similar for all groups. The rats were given subcutaneous injections (0.1 ml/rat) for 8 weeks (days 1-56) of vehicle, estradiol (10 μg/kg) and/or PEG-rTGF-β2 (1 μg/kg). PEG-rTGF-β2 was given every day for 3 weeks, then 3 times a week for 4 weeks, and then every day during the last week. The PEG-rTGF-β2 vehicle was 40% propylene glycol with <2% ethanol in PBS with pH adjusted to 7.2. The study groups were organized as follows:

Group	Injection(s)
sham	PEG-rTGF-β2 vehicle
OVX	None - sacrificed at the start of the study
OVX	PEG-rTGF-β2 vehicle
OVX	PEG-TGF-β2 vehicle + estradiol (10 μg/kg)
OVX	PEG-rTGF-β2 (1 μg/kg)
OVX	PEG-rTGF-β2 (1 μg/kg)+ estradiol (10 μg/kg)

All values below show means ± standard error of the mean, with number of rats in parentheses.

Table 1

Initial Body Weight (g) and Spine BMD (mg/cm^2) Values

Treatment	Body Weight	Spine BMD
Sham	411±9(4)	252±5(4)
Pretreatment OVX	361±6(7)	204±6(7)
OVX + Vehicle	371±8(10)	202±4(10)
Estradiol	376±12(7)	204±5(7)
PEG-rTGF-β2	363±7(10)	204±16(6)
PEG-rTGF-β2 + Estradiol	363±10(11)	198±13(7)

For each rat, the individual body weights for days 21, 42 and 56 were subtracted from the day 0 value to obtain the individual changes and are shown in Table 2 below.

Table 2

Body Weight Changes (grams)
during the Course of the Study

Treatment	3 Weeks	6 Weeks	8 Weeks
Sham	-19±7(4)	-12±6(4)	-32±12(4)
OVX + Vehicle	-4±3(10)	-2±5(10)	-5±4(10)
Estradiol	-12±3(7)	-25±7(7)	-28±6(6)
PEG-rTGF-β2	-6±5(10)	-3±7(10)	-11±10(10)
PEG-rTGF-β2 + Estradiol	-17±4(11)	-18±7(11)	-27±7(11)

All rats were scanned at the beginning and at 3, 6, and 9 weeks into the study. For each rat the difference between their individual spine BMD values (mg/cm^2) at 3, 6 and 9 weeks and the initial values measured on day 0 value were calculated to obtain the individual changes in spine BMD reported in Table 3.

Table 3

Spine BMD Changes during the Course of the Study

Treatment	3 Weeks	6 Weeks	8 Weeks
Sham	5±9(4)	2±6(4)	2±9(4)
OVX + Vehicle	5±5(10)	1±4(10)	2±5(10)
Estradiol	6±4(7)	5±5(7)	6±4(6)
PEG-rTGF-β2	-16±9(6)	-11±7(6)	-18±7(6)
PEG-rTGF-β2 + Estradiol	3±3(7)	3±5(7)	5±4(7)

After sacrifice at 8 weeks, the tibia were removed and scanned. The tibia lengths and projected areas are presented in Table 4. The tibia bone mineral content, bone mineral density and bone mineral apparent density data are given in Table 5. The bone mineral densities for tibial cortical bone, metaphysis and epiphysis are given in Table 6.

Table 4

Tibia Lengths and Projected Areas

Treatment	N	Length(mm)	Area (cm^2)
Sham	4	42.8±0.4	1.45±0.05
Pretreatment OVX	7	43.0±0.5	1.49±0.03
OVX + Vehicle	10	42.4±0.4	1.45±0.02
Estradiol	7	42.3±0.2	1.44±0.01
PEG-rTGF-β2	10	42.0±0.3	1.41±0.02
PEG-rTGF-β2 + Estradiol	11	42.1±0.3	1.45±0.03

Table 5

Tibia Global BMC, BMD and BMAD Values

Treatment	N	BMC (mg)	BMD (mg/cm^2)	BMAD (mg/cm^3)
Sham	4	335±25	230±12	191±9
Pretreatment OVX	7	311±11	209±4	171±3
OVX + Vehicle	10	303±7	209±2	174±1
Estradiol	7	312±9	217±5	180±4
PEG-rTGF-β2	10	293±12	207±6	174±4
PEG-rTGF-β2 + Estradiol	11	303±13	208±6	173±4

Table 6

Tibia Cortical Bone, Metaphysis and Epiphysis BMD Values

Treatment	N	Cortical Bone	Metaphysis (mg/cm^2)	Epiphysis (mg/cm^3)
Sham	4	238±8	217±27	266±25
Pretreatment OVX	7	204±4	209±3	209±6
OVX + Vehicle	10	203±3	205±3	210±4
Estradiol	7	208±6	219±7	226±8
PEG-rTGF-β2	10	199±6	204±7	214±9
PEG-rTGF-β2 + Estradiol	11	199±6	209±7	215±9

Table 7 indicates that the estradiol dose was sufficient to normalize the uterine size, but PEG-rTGF-β2 had little if any effect.

Table 7

Uterine Weight (g) at Sacrifice

Treatment	N	Uterine Weight
Sham	4	0.85±0.11
OVX + Vehicle	10	0.16±0.02
Estradiol	6	0.80±0.13
PEG-rTGF-β2	10	0.25±0.08
PEG-rTGF-β2 + Estradiol	10	0.60±0.05

It appeared that on sacrifice there was less cutaneous fibrosis with PEG-rTGF-β2 than with TGF-β2. Less fibrosis at the injection site is an important clinical advantage, because fibrosis can interfere with absorption and cause unsightly scar-like tissue.

Dose Effect of Recombinant TGF-β2-$(PEG\ 5000)_6$ on Bone Remodeling in Bilaterally Ovariectomized Adult Rats

Recruitment and increased activity of osteoblast cell population was observed in previous experiments on normal mice which had been treated with subcutaneous administration of PEG-TGF-β2. In the eleven-day mouse study, all measured bone formation parameters were increased; whereas, bone resorption parameters remained unchanged. The net result was an increase in trabecular bone mass in the group of animals treated with 0.4μg/kg body weight of rTGF-β2-$(PEG\ 5000)_6$ injected subcutaneously over an 11-day period.

This purpose of this study is to:

a) test the ability of rTGF-β2$(PEG\ 5000)_6$ to promote bone formation in osteopenic rat skeleton after bilateral ovariectomy;

b) compare effects on bone of three different doses of PEG-TGF-β2 when injected daily (versus the effect of the same dose injected on a twice-a-week basis); and

c) compare dose response of PEG-TGF-β2 administration on bone-forming and bone-resorbing cells over the two- and four-week periods.

One hundred and thirty five female, Sprague-Dawley rats are obtained at 90 days of age and housed for one week to acclimate. After acclimation, 23 randomly chosen animals are sham-operated; whereas, the remaining 112 females are bilaterally ovariectomized by the dorsal approach under ketamine/xylazine anesthesia. Eight weeks post sham surgery or ovariectomy, animals are randomly divided in eight experimental groups and are treated as shown:

Group	Treatment	Frequency of treatment	Number of Animals Euthanized at Each Time Point		
			day 0	day 14	day 28
1. Sham	vehicle	daily	5	9	9
2. OVX	vehicle	daily	5	9	9
3. OVX	5μg/kg	daily	0	7	7
4. OVX	1μg/kg	daily	0	7	7
5. OVX	0.2μg/kg	daily	0	7	7
6. OVX	5μg/kg	2/week	0	7	7
7. OVX	1μg/kg	2/week	0	7	7
8. OVX	0.2μg/kg	2/week	0	7	7

Five animals from groups 1 and 2 serve as baseline controls and show bone status in sham and OVX groups before treatment with PEG-TGF-β2.

Seven to nine animals from each group are sacrificed 14 and 28 days after treatment started. All animals receive intraperitoneal injections of fluorescent bone markers: calcein (10 mg/kg) is given at day 0 and day 11 to animals euthanized at day 14 and at day 14 and day 21 to animals sacrificed at day 28. Declomycin (25 mg/kg) is administered at day 7 to animals scheduled to be sacrificed on day 14 and on

day 21 to animals scheduled to be sacrificed on day 28.

Body weights are monitored for all animals weekly. Animals are necropsied by exsanguination from the vena cava under deep anesthesia with ketamine/xylazine.

Livers from the Group 3 mice killed on day 28 are processed for histopathology.

Both femurs, tibias, lumbar vertebral bodies, and mandibles are collected, fixed in ethanol and processed for various histomorphometric analyses. Distal femoral metaphyses from right femurs are embedded undecalcified for static and dynamic histomorphometry of cancellous bone. Also, cortical bone histomorphometry is performed on cross-sections from right tibias at the tibio-fibular junction. The proximal portion of the same tibias is demineralized, embedded in paraffin, cut in thin longitudinal sections (4-5μm). These sections are used for osteoid and cellular measurements (osteoid surface, osteoid maturation time, osteoblast surface, osteoclast surface, osteoclast number, and number of nuclei per osteoclast). Bodies of lumbar vertebrae (L5 and L6) are embedded decalcified and serve for histomorphometric measurements of cancellous bone.

Left tibias which have been cleaned of soft tissues are used for determination of wet, dry and ash weight. Also, if necessary, calcium and phosphorus are determined from ash. Left femurs and mandibles are stored undecalcified at -70°C for eventual additional measurements.

Other Embodiments

Other embodiments are within the following claims.

Claims

1. A method for stimulating bone formation in an animal, comprising the step of administering to the animal an effective amount of a conjugate of a growth factor and a hydrophilic polymer.

2. The method of claim 1 wherein said growth factor is a TGF-β.

3. The method of claim 2 wherein said growth factor is a recombinant TGF-β.

4. The method of claim 2 wherein said growth factor is a TGF-β2.

5. The method of claim 2 wherein said growth factor is a recombinant TGF-β2.

6. The method of claim 1 wherein said hydrophilic polymer has an affinity for bone *in vivo*.

7. The method of claim 1 wherein said hydrophilic polymer comprises a polyethylene glycol.

8. The method of claim 7 wherein said polyethylene glycol has a molecular weight in the range of about 200 daltons to about 100,000 daltons.

9. The method of claim 8 wherein said polyethylene glycol has a molecular weight of about 5000 daltons.

10. The method of claim 7 wherein said polyethylene glycol has a molecular weight of about 35,000 daltons.

11. The method of claim 1 wherein said polyethylene glycol has a molecular weight of about 55,000 daltons to about 130,000 daltons.

12. The method of claim 1 wherein said hydrophilic polymer comprises a polypropylene glycol.

13. The method of claim 1 wherein the hydrophilic polymer and the growth factor in said conjugate are in a molar ratio in the range of about 1:1 to about 14:1.

14. The method of claim 13 wherein the hydrophilic polymer and the growth factor in said conjugate are in a molar ratio in the range of about 1:1 to about 7:1.

15. The method of claim 10 wherein the growth factor is a TGF-β and wherein the polyethylene glycol and the TGF-β in said conjugate are in a molar ratio in the range of about 4:1 to about 7:1.

16. The method of claim 11 wherein the growth factor is a TGF-β and wherein the polyethylene glycol and the TGF-β in said conjugate are in a molar ratio in the range of about 1:1 to about 4:1.

17. The method of claim 1, further comprising administering to the animal a bone resorption inhibitor.

18. The method of claim 17 wherein said bone resorption inhibitor comprises an estrogen.

19. The method of claim 17 wherein said bone resorption inhibitor comprises a bisphosphonate.

20. The method of claim 17 wherein said bone resorption inhibitor comprises a calcitonin.

21. The method of claim 17 wherein said growth factor — hydrophilic polymer conjugate is administered at least partially concurrently.

22. A composition for promoting bone formation *in vivo*, comprising a conjugate of a growth factor and a hydrophilic polymer.

23. The composition of claim 22 wherein said hydrophilic polymer has an affinity for bone *in vivo*.

24. The composition of claim 22 wherein said growth factor is a TGF-β.

25. The composition of claim 24 wherein said growth factor is a recombinant TGF-β.

26. The composition of claim 24 wherein said growth factor is dimer containing a TGF-β2 subunit.

27. The composition of claim 25 wherein said growth factor is a recombinant TGF-β2.

28. The composition of claim 27 wherein said growth factor is a recombinant human TGF-β2.

29. The composition of claim 19 wherein said hydrophilic polymer comprises a polyethylene glycol.

30. The composition of claim 29 wherein said polypropylene glycol has a molecular weight between about 200 daltons and about 100,000 daltons.

31. The composition of claim 30 wherein said polyethylene glycol has a molecular weight of about 5000 daltons.

32. The composition of claim 30 wherein said polyethylene glycol has a molecular weight of about 1700 daltons.

33. The composition of claim 30 wherein said polyethylene glycol has a molecular weight of about 35,000 daltons.

34. The composition of claim 22 wherein the growth factor is a TGF-β and wherein the polyethylene glycol and the TGF-β in said conjugate are in a molar ratio in the range of about 1:1 to about 4:1.

35. The composition of claim 22, further comprising a bone resorption inhibitor.

36. The composition of claim 35 wherein said bone resorption inhibitor comprises an estrogen.

37. The composition of claim 35 wherein said bone resorption inhibitor comprises a bisphosphonate.

38. The composition of claim 35 wherein said bone resorption inhibitor comprises a calcitonin.

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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

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APS; DIALOG: MEDLINE; WPI
SEARCH TERMS: TGF-BETA, BONE RESORPTION, BONE GROWTH, HYDROPHILIC POLYMER

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US, A, 5,208,219 (OGAWA ET AL) 04 MAY 1993, COLUMNS 5-6; COL. 7, LINES 36-41; COL. 8, LINES 64-68.	1-38
Y	US, A, 5,118,667 (ADAMS ET AL) 02 JUNE 1992, COL. 3, LINES 27-35.	1-38
Y	US, A, 4,179,337 (DAVIS ET AL) 18 DECEMBER 1979, COL. 2, LINES 53-58.	1-38
Y	US, A, 5,162,430 (RHEE ET AL) 10 NOVEMBER 1992, COL. 5.	1-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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(54) Title: CELL GROWTH SUBSTRATES WITH TETHERED CELL GROWTH EFFECTOR MOLECULES

(57) Abstract

Disclosed are compositions with tethered growth effector molecules, and methods of using these compositions for growing cells and tissues. Growth effector molecules, including growth factors and extracellular matrix molecules, are flexibly tethered to a solid substrate. The compositions can be used either *in vitro* or *in vivo* to grow cells and tissues. By tethering the growth factors, they will not diffuse away from the desired location. By making the attachment flexible, the growth effector molecules can more naturally bind to cell surface receptors. A significant feature of these compositions and methods is that they enhance the biological response to the growth factors. The new method also offers other advantages over the traditional methods, in which growth factors are delivered in soluble form: (1) the growth factor is localized to a desired target cell population; (2) significantly less growth factor is needed to exert a biologic response. This method can be used as a means of enhancing the therapeutic use of growth factors *in vivo* and of creating surfaces which will enhance *in vitro* growth of difficult-to-grow cells such as liver cells.

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CELL GROWTH SUBSTRATES WITH TETHERED CELL GROWTH EFFECTOR MOLECULES

Background Of The Invention

This invention concerns cell and tissue growth substrates, growth stimulation compositions, and methods for delivering growth factors to cells and tissues.

Long-term mammalian cell culture has been difficult to achieve. Many types of specialized cells plated on standard tissue culture plastic dishes dedifferentiate, lose function, and fail to proliferate. There are many applications of mammalian cell culture that could benefit from methods or materials which enhance the long term stability of differentiated mammalian cells in culture. These cells are currently used as sources of natural and engineered proteins and glycoproteins, in screens for the effects of compounds on cell proliferation and function, and for implantation to supplement or replace cell function. Certain cells are particularly difficult to maintain in long term culture, such as hepatocytes.

It would be especially useful if hepatocytes could be maintained in long term culture. For example, *in vitro* toxicity testing of ingestible of orally administered compounds has been hampered by the fact that the liver converts many compounds into other chemical forms. These other forms may be toxic or have other effects. Thus, complete testing of materials in cell culture must include the effects of biotransformations carried out by the liver. Using current methodology, it is difficult to grow normal liver cells *in vitro* beyond two to three cell divisions. The result is that *in vitro* testing does not reduce the number of animals needed because essentially all of the cells to be used *in vitro* must come from direct isolation. A method of expanding liver cells *in vitro* would make it feasible to use *in vitro* liver cell cultures to carry out biotransformations by applying the compound of interest directly to liver cells in culture. The supernate from the liver cell cultures

could then be applied to other types of cells, such as skin, lung, nerve, and bladder, to assess the effect of the metabolized compound of interest.

Studies have been conducted for a number of years to improve the viability, proliferation and differentiated function of eukaryotic cells cultured *in vitro*. One discovery has been the importance of extracellular matrix and extracellular matrix molecules in maintaining cell function and allowing cell growth. These effects, and methods of using matrix components for cell growth, have been described by, for example, Jauregui *et al.*, *In Vitro Cellular & Developmental Biology* **22**: 13-22 (1986), Kleinman *et al.*, *Analytical Biochemistry* **166**: 1-13 (1987), and Mooney *et al.*, *Journal of Cellular Physiology* **151**: 497-505 (1992).

Growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factors (TGFα, TGFβ), exert a broad mitogenic response. Growth factors and their effects have been described in "Peptide Growth Factors and Their Receptors I" M.B. Sporn and A.B. Roberts, eds. (Springer-Verlag, New York, 1990). In recognition of their importance, most cell and tissue growth compositions include growth factors, either as an additive or as a component of complex growth media. The use of growth factors in this manner has certain drawbacks. For example, cells have a complex, nonlinear response to the concentration of growth factor in their environment. Extended exposure to high growth factor concentrations may cause cells to lose responsiveness to the factor. For example, EGF, a potent mitogen for a wide variety of cell types and arguably the best-characterized of the growth factors, when delivered in soluble form, is typically internalized by the cell, and the cell often responds by down-regulating the number of EGF receptors. This down-regulation causes cells to lose responsiveness to EGF.

Growth factors have also been used in disappointingly few clinical products, considering the range of effects they produce *in vitro*. Translation

of the mitogenic effects observed for the target cell *in vitro* to tissue growth *in vivo* is hampered by several issues. For example, the growth factors, when placed in a complex cellular environment, often end up stimulating the growth of competing cells which then overgrow the target cells. Researchers have attempted to solve this problem by targeting delivery of factors at a specific site, but this approach is not always successful because soluble growth factors can readily diffuse into the blood stream and away from the target site, exerting their effects elsewhere. This diffusion of growth factors is also a problem because it increases the amount of growth factor that must be used in order to have the desired local effect. Internalization of growth factors and loss of responsiveness to growth factors is a particular problem for *in vivo* applications considering the amount of time cell growth must be stimulated to allow wound healing.

Another attempt to improve the longevity of growth factor effects *in vivo* has been to incorporate growth factors in a slow release material. Such a scheme still requires large amounts of growth factor and does not address the problem of competing cell growth due to diffusion of the growth factors. The large amount of growth factors needed for these cell and tissue growth methods is a particular problem because growth factors are difficult and expensive to prepare.

It is therefore an object of the invention to provide a cell and tissue growth substrate that stimulates long-term target cell growth.

It is another object of the invention to provide a tissue growth scaffold for growth of a target tissue *in vivo*.

It is a further object of the invention to provide a method of long-term cell and tissue growth *in vitro*, and to provide a method of growing target tissue *in vivo*.

It is another object of the invention to provide an *in vitro* tissue analog for drug and toxicity testing, and a method of drug and toxicity testing using the tissue analog.

Summary Of The Invention

The methods and compositions described herein concern new cell and tissue growth substrates. Growth effector molecules, including growth factors and extracellular matrix molecules, are flexibly tethered to a support medium and the combination is used to stimulate and support cell and tissue growth. The most significant feature of these compositions is that they enhance the biological response to the growth factor. The new compositions also offers other advantages over the traditional growth methods, in which growth factors are delivered in soluble form: (1) the growth factor is localized to a desired target cell population, and (2) significantly less growth factor is needed to exert a biologic response. In a preferred embodiment, multiple growth factors and/or matrix materials are attached to a single core molecule, such as a star polymer. These compositions can be used as a means of enhancing the therapeutic use of growth factors *in vivo* and of creating surfaces which will enhance *in vitro* growth of difficult-to-grow cells such as liver cells.

Brief Description Of The Drawings

Figure 1 is a graph of DNA synthesis in cells grown on a non-tethered substrate, with EGF present or absent from the growth medium, plotting labeling index versus the presence or absence of EGF. The labeling index is the percentage of cells in a field that have stained nuclei.

Figure 2 is a graph of DNA synthesis in cells grown with tethered (coupled) or adsorbed EGF, plotting labeling index versus tethered or

adsorbed EGF. The labeling index is the percentage of cells in a field that have stained nuclei.

Detailed Description Of The Invention

Many problems with effective utilization of growth factors may be overcome if, instead of being delivered in soluble form, the growth factors are immobilized on a solid substrate. This approach is attractive because some forms of insoluble matrix, such as crosslinked collagen sponges and bioresorbable polyester fabric, are used for many types of tissue regeneration to provide a template for tissue growth. The solid support need not be permanent, and thus the approach may be used for almost any tissue. Immobilization prevents the factor from diffusing away from the site and consequently allows a much more highly targeted form of delivery than other methods. Besides this concentration effect, tethering has other powerful advantages, stemming from the way growth factors work. For example, when delivered in soluble form, EGF is typically internalized by the cell, and the cell often responds by down-regulating the number of EGF receptors. However, evidence now shows that the growth factor does not have to be internalized in order to stimulate cell growth. For example, Reddy *et al.*, *Biotechnology Progress* **10**: 377-384 (1994), describes fibroblasts that remain responsive to EGF despite their expression of internalization-deficient EGF receptors. As demonstrated by the following example, by allowing the target cell to bind EGF, but preventing the cell from internalizing the bound EGF, it is possible to circumvent the normal down-regulation of receptors that occurs in the presence of high concentrations of EGF. This offers two advantages: (1) it is possible to speed the rate of target cell growth *in vivo*, because cells in contact with the surface bearing the growth factor do not lose their responsiveness to EGF, and (2) considerably less growth factor is required, because cells do not internalize and degrade the growth factor. The

method of attachment of the growth factor to the substrate is critical because the receptor must have access to the factor. Furthermore, for some growth factors, dimerization or aggregation in the membrane is believed to be critical, as described in "Peptide Growth Factors and Their Receptors I" M.B. Sporn and A.B. Roberts, eds. (Springer-Verlag, New York, 1990). Thus, the growth factor will either have to be immobilized in extremely high concentration or immobilized on flexible tethers which will allow the ligand-receptor complex to aggregate in the cell membrane. Direct immobilization of even high concentrations of growth factor may be ineffective if the receptors bind randomly.

Tethers

Requirements

As used herein, a tether is a flexible link between an attachment substrate and a growth effector molecule. Flexible tethers for attaching growth effector molecules to a substrate must satisfy two important requirements: (1) the need for mobility of the ligand-receptor complex within the cell membrane in order for the effector molecule to exert an effect, and (2) biocompatibility of materials used for immobilization. Substantial mobility of a tethered growth factor is critical because even though the cell does not need to internalize the complex formed between the receptor and the growth factor, it is believed that several complexes must cluster together on the surface of the cell in order for the growth factor to stimulate cell growth. In order to allow this clustering to occur, the growth factors are attached to the solid surface, for example, via long water-soluble polymer chains, which are referred to as tethers, allowing movement of the receptor-ligand complex in the cell membrane.

Examples of water-soluble, biocompatible polymers which can serve as tethers include polymers such as synthetic polymers like polyethylene oxide (PEO), polyvinyl alcohol, polyhydroxyethyl methacrylate, polyacrylamide,

and natural polymers such as hyaluronic acid, chondroitin sulfate, carboxymethylcellulose, and starch.

Tethers can also be branched to allow attachment of multiple growth effector molecules in close proximity. Branched tethers can be used, for example, to increase the density of growth effector molecule on the substrate. Such tethers are also useful in bringing multiple or different growth effector molecules into close proximity on the cell surface. This is useful when using a combination of different growth effector molecules. Preferred forms of branched tethers are star PEO and comb PEO.

Star PEO is formed of many PEO "arms" emanating from a common core. Star PEO has been synthesized, for example, by living anionic polymerization using divinylbenzene (DVB) cores, as described by Gnanou *et al.*, *Makromol. Chemie* **189**: 2885-2892 (1988), and Merrill, *J. Biomater. Sci. Polymer Edn* **5**: 1-11 (1993). The resulting molecules have 10 to 200 arms, each with a molecular weight of 3,000 to 12,000. These molecules are about 97% PEO and 3% DVB by weight. Other core materials and methods may be used to synthesize star PEO. Comb PEO is formed of many PEO chains attached to and extending from the backbone of another polymer, such as polyvinyl alcohol. Star and comb polymers have the useful feature of grouping together many chains of PEO in close proximity to each other.

Length

The length of a tether is limited only by the mechanical strength of the tether used and the desired stability of a tethered growth factor. It is expected that stronger tethers can be made longer than weaker tethers, for example. It is also desirable for tether length and strength to be matched to give a desired half life to the tether, prior to breakage, and thereby adjust the half life of growth factor action. The minimum tether length also depends on the nature of the tether. A more flexible tether will function well even if the tether length is relatively short, while a stiffer tether may need to be longer to allow effective contact between a cell and the growth effector molecules.

The backbone length of a tether refers to the number of atoms in a continuous covalent chain from the attachment point on the substrate to the attachment point of the growth effector molecule. All of the tethers attached to a given substrate need not have the same backbone length. In fact, using tethers with different backbone lengths on the same substrate can make the resulting composition more effective and more versatile. In the case of branched tethers, there can be multiple backbone lengths depending on where and how many growth effector molecules are attached. Preferably, tethers can have any backbone length between 5 and 50,000 atoms. Within this preferred range, it is contemplated that backbone length ranges with different lower limits, such as 10, 15, 25, 30, 50, and 100, will have useful characteristics.

Such tethers are not intended to be limited by the manner in which the substrate-tether-growth effector molecule composition is assembled. For example, if linker molecules are attached to the substrate and the growth effector molecule, and then the linkers are joined to form the tethered composition, the entire length of the joined linkers is considered the tether. As another example, the attachment substrate may, by its nature, have on its surface protruding molecular chains. If a linker molecule is attached to the substrate via such protruding chains, then the chain and linker together are considered to be a tether.

Biocompatible polymers and spacer molecules are well known in the art and most are expected to be suitable for forming tethers. The only important characteristics are biocompatibility and flexibility. That is, the tether should not be made of a substance that is cytotoxic or, in the case of *in vivo* uses, which causes significant allergic or other physiological reaction when implanted. The tether should also allow the growth factor a sufficient range of motion to effectively bind to a cell surface receptor.

The biodegradability of a tether, the tether-substrate link, or the tether-growth factor link can be used to regulate the length of time a growth factor

stimulates growth. For example, if a given tether degrades during cell growth at a consistent rate, then a limit can be placed on how long the growth factors binds to and stimulates cell growth. Once untethered, a growth factor can be internalized by the cell or can diffuse away from the target cells. Such planned degradation is especially useful in the context of implanted compositions, used to stimulate tissue replacement, by limiting the amount of tissue growth.

Attachment Substrates

There are two basic types of substrates onto which growth effector molecules can be tethered. One class includes biocompatible materials which are not biodegradable, such as polystyrenes, polyethylene vinyl acetates, polypropylenes, polymethacrylates, polyacrylates, polyethylenes, polyethylene oxides, glass, polysilicates, polycarbonates, polytetrafluoroethylene, fluorocarbons, nylon, silicon rubber, and stainless steel alloys. The other class of materials includes biocompatible, biodegradable materials such as polyanhydrides, polyglycolic acid, polyhydroxy acids such as polylactic acid, polyglycolic acid, and polylactic acid-glycolic acid copolymers, polyorthoesters, polyhydroxybutyrate, polyphosphazenes, polypropylfumerate, and biodegradable polyurethanes, proteins such as collagen and polyamino acids, and polysaccharides such as glycosaminoglycans, alginate, and carageenan, bone powder or hydroxyapatite, and combinations thereof. These biodegradable polymers are preferred for *in vivo* tissue growth scaffolds. Other degradable polymers are described by Engleberg and Kohn, *Biomaterials* 12: 292-304 (1991).

Attachment substrates can have any useful form including bottles, dishes, fibers, woven fibers, shaped polymers, particles and microparticles. For *in vitro* cell growth, the growth effector molecule can be tethered to standard tissue culture polystyrene petri dishes. Woven fibers are useful for stimulating growth of tissue in the form of a sheet, sponge or membrane.

The biodegradability of a substrate can be used to regulate the length of time the growth factor stimulates growth and to allow replacement of implanted substrate with new tissue. For this purpose the substrate with tethered growth effector molecules can be considered a scaffold upon which new tissue can form. As such, a degradable scaffold is broken down as tissue replacement proceeds. Once released from the substrate, a growth factor can be internalized or can diffuse away from the target cells. Such planned degradation is especially useful in the context of implanted compositions, used to stimulate tissue replacement, by limiting the amount of tissue growth and eliminating the need to remove the tissue scaffold. For implantation in the body, preferred degradation times are typically less than one year, more typically in the range of weeks to months.

In some embodiments, attachment of the cells to the substrate is enhanced by coating the substrate with compounds such as extracellular membrane components, basement membrane components, agar, agarose, gelatin, gum arabic, collagen types I, II, III, IV, and V, fibronectin, laminin, glycosaminoglycans, mixtures thereof, and other materials known to those skilled in the art of cell culture.

Growth Effector Molecules

Growth effector molecules, as used herein, refer to molecules that bind to cell surface receptors and regulate the growth, replication or differentiation of target cells or tissue. Preferred growth effector molecules are growth factors and extracellular matrix molecules. Examples of growth factors include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factors (TGFα, TGFβ), hepatocyte growth factor, heparin binding factor, insulin-like growth factor I or II, fibroblast growth factor, erythropoietin, nerve growth factor, bone morphogenic proteins, muscle morphogenic proteins, and other factors known to those of skill in the art. Additional growth factors are described in "Peptide Growth

Factors and Their Receptors I" M.B. Sporn and A.B. Roberts, eds. (Springer-Verlag, New York, 1990), for example.

Growth factors can be isolated from tissue using methods know to those of skill in the art. For example, growth factors can be isolated from tissue, produced by recombinant means in bacteria, yeast or mammalian cells. For example, EGF can be isolated from the submaxillary glands of mice and Genentech produces TGF-β recombinantly. Many growth factors are also available commercially from vendors, such as Sigma Chemical Co. of St. Louis, MO, Collaborative Research, Genzyme, Boehringer, R&D Systems, and GIBCO, in both natural and recombinant forms.

Examples of extracellular matrix molecules include fibronectin, laminin, collagens, and proteoglycans. Other extracellular matrix molecules are described in Kleinman *et al.* (1987) or are known to those skilled in the art. Other growth effector molecules useful for tethering include cytokines, such as the interleukins and GM-colony stimulating factor, and hormones, such as insulin. These are also described in the literature and are commercially available.

The specific function or effect of a growth effector molecule does not limit its usefulness in the disclosed compositions and methods. This is because tethering of a growth effector molecule is used to prevent loss of effect caused by diffusion away from a target cell and/or internalization of a growth factor.

Only those growth effector molecules that can exert an effect while tethered are useful in the disclosed compositions. Such an effect, however, need not be the same effect or require the same concentration as the untethered growth effector molecule. So long as a growth effector molecule can exert any desired growth effect on a cell while tethered it is considered to be useful for tethering. These useful effects can be determined by tethering a selected growth effector molecule and observing the effect on cell growth using growth assays, such as those described in the examples below.

Attachment Methods

Standard immobilization chemistries, which are well known in the art, can be used to covalently link the tethers to the growth effector molecule and the substrate. Tethering growth effector molecules can be accomplished by attachment, for example, to aminated surfaces, carboxylated surfaces or hydroxylated surfaces using standard immobilization chemistries. Examples of attachment agents are cyanogen bromide, succinimide, aldehydes, tosyl chloride, avidin-biotin, photocrosslinkable agents, epoxides and maleimides. A preferred attachment agent is glutaraldehyde. These and other attachment agents, as well as methods for their use in attachment, are described in "Protein immobilization: fundamentals and applications" Richard F. Taylor, ed. (M. Dekker, New York, 1991). Growth effector molecules can be tethered to a substrate by chemically cross-linking a tether molecule to reactive side groups present within the substrate and to a free amino group on the growth effector molecule. For example, synthetic EGF may be chemically cross-linked to a substrate that contains free amino or carboxyl groups using glutaraldehyde or carbodiimides as cross-linker agents. In this method, aqueous solutions containing free tethers molecules are incubated with the substrate in the presence of glutaraldehyde or carbodiimide. For crosslinking with glutaraldehyde the reactants can be incubated with 2% glutaraldehyde by volume in a buffered solution such as 0.1 M sodium cacodylate at pH 7.4. Other standard immobilization chemistries are known by those of skill in the art and can be used to join substrates, tethers, and growth effector molecules.

For the disclosed cell growth compositions, growth effector molecules may be tethered either alone or in combinations. For example, both insulin and EGF may be tethered to the same substrate. The growth effector molecules may be combined in any desired proportions. The relative amounts of different growth effector molecules can be controlled, for example, by first separately linking the growth effector molecules to tethers,

then mixing the "loaded" tethers in the desired proportions and attaching them to the substrate. The proportion of each growth effector molecule tethered to the substrate should match the proportion of loaded tethers in the attachment reaction.

Tethering to Aminated Surfaces.

Cell culture surfaces bearing primary amines can be prepared, for example, by amino-siloxane treatment of glass using reagents which can be commercially purchased and applied to standard laboratory glassware or by plasma discharge treatment of polymers in an ammonia environment. Collagen matrices for tissue regeneration have primary amines present in lysine side chains and the terminal amines of each molecule. Two approaches are possible. Polymers such as PEO tethers can be activated on both ends with a leaving group such as tresyl chloride which reacts with primary amines. No blocking is necessary because only the terminal hydroxyl residues of tether are reactive. This type of reaction can be carried out using standard glassware in a chemical fume hood. Vacuum drying of the product is required as an intermediate step. A substantial excess of the activated tether over the number of available amines, dissolved in a saline buffer, is added to the surface to be modified and the coupling reaction is allowed to proceed. Use of an excess of activated PEO in this step minimizes the reaction of both ends of PEO with available amines and ensure a substantial fraction of unreacted activated chain ends are left for reaction with the growth factor. Unreacted PEO is washed away, and the EGF is then added in saline solution to react with the remaining activated chain ends. If mouse EGF is used, only the terminal amino acid is reactive because it contains no other primary amines. Human EGF contains three possible immobilization sites. After the reaction is completed, excess unreacted growth factor is removed. This first approach is preferred for attaching EGF to a matrix such as crosslinked collagen, which contains a large number of

free hydroxyls and which does not allow significant non-specific adsorption of EGF.

A second approach is to activate the tethers on only one end initially by using a substoichiometric amount of activating agent. This will yield a distribution of species which include completely unactivated tether as well as tether activated at both ends. Unactivated tether can easily be washed away after the attachment step. The tether is then coupled to the support as described above, and the free tether ends are then activated to allow attachment of EGF. This second approach is preferred for derivatization of cell culture surfaces, which might allow substantial non-specific adsorption of growth factor, because an intermediate step in which unreacted amines are blocked with short-chain monomethoxy PEO can be added before EGF attachment in order to minimize non-specific adsorption of the factor.

Cells

Cells to be cultured using the disclosed compositions can be any cells that respond to growth factors or that need growth effector molecule for growth. For example, cells can be obtained from established cell lines or separated from isolated tissue. Cells types that can be used with the tethered growth effector molecule compositions include most epithelial and endothelial cell types, for example, parenchymal cells such as hepatocytes, pancreatic islet cells, fibroblasts, chondrocytes, osteoblasts, exocrine cells, cells of intestinal origin, bile duct cells, parathyroid cells, thyroid cells, cells of the adrenal-hypothalamic-pituitary axis, heart muscle cells, kidney epithelial cells, kidney tubular cells, kidney basement membrane cells, nerve cells, blood vessel cells, cells forming bone and cartilage, and smooth and skeletal muscle. The cells used can also be recombinant. Methods for gene transfer are well known to those skilled in the art.

In Vitro Cell And Tissue Growth Using Substrates With Tethered Growth Effector Molecules

Substrates with tethered growth effector molecules can be used to improve *in vitro* culture of hard-to-grow cells such as liver cells. Liver cell cultures would be useful for toxicology testing to replace certain aspects of animal testing of drugs. Liver cells grow very poorly *in vitro* using prior art methods, typically undergoing only one or two rounds of DNA synthesis after they are placed in culture. Since a tethered growth factor cannot be internalized, tethering will change the way the cells respond to the factor, constantly stimulating them to grow.

Cells can be cultured with tethered growth effector molecule compositions using any of the numerous well known cell culture techniques. Standard cell culture techniques are described in Freshney, "Cell Culture, a manual of basic technique" Third Edition (Wiley-Liss, New York, 1994). Other cell culture media and techniques well known to those skilled in the art can be used with the disclosed compositions. The disclosed compositions are adaptable to known cell culture vessels. For example, growth effector molecules can be immobilized on standard tissue culture polystyrene and glass petri dishes, T-flasks, roller bottles, stackable chambers, and filter systems such as the Millipore MILLICELL™ inserts, hollow fiber reactors and microcarriers. Cells can also be cultured in suspension using the disclosed compositons by tethering growth effector molecules to tiny beads or fibers, on the order of 10 microns in diameter of length. Such tiny particles, when added to culture medium, would attach to cells thereby stimulating their growth and providing attachment signals. The only critical difference in culturing technique is the elimination of growth factor from the cell culture medium when using tethered growth factor compositions. As described in the examples below, using soluble versus tethered EGF in primary hepatocyte cultures show an enhanced DNA synthesis rate of the tethered growth factor

in comparison to the soluble growth factor. This effect is dependent on the amount of the immobilized factor.

***In Vivo* Tissue Growth Using Tissue Growth Scaffolds With Tethered Growth Effector Molecules**

In yet another embodiment of the present invention, erodible and non-erodible artificial matrices with tethered growth effector molecules may be used either alone or in combination with attached cells to remodel tissue architecture or to repair tissue defects and wounds.

Known methods and compositions for culturing cells and implanting them into the body can be adapted to use tethered growth effector molecules. For example, U.S. Patent No. 4,352,883 to Lim, uses cells that are encapsulated within alginate microspheres, then implanted. Such microspheres can be modified with tethered growth effector molecules to improve their usefulness. Culturing cells on a matrix for use as artificial skin, as described by Yannas and Bell in a series of publications, can also be modified by tethering growth effector molecules to the matrix. U.S. Patent No. 4,485,097 to Bell, U.S. Patent No. 4,060,081 to Yannas *et al.*, and U.S. Patent No. 4,458,678 to Yannas *et al.* describe substrates for use as artificial skin. U.S. Patent No. 4,520,821 to Schmidt describes a similar approach that was used to make linings to repair defects in the urinary tract.

Vacanti *et al.*, *Arch. Surg.* **123**: 545-549 (1988), describes a method of culturing dissociated cells on biocompatible, biodegradable matrices for subsequent implantation into the body. Cima and Langer, "Tissue Engineering" *Chem. Eng. Prog.* **89**: 46-54 (1993), describe important considerations for the nature and form of implanted matrices useful for inducing tissue replacement. U.S. Patent Application Serial No. 08/200,636 entitled "Tissue Regeneration Matrices by Solid Free Force Fabrication" filed February 23, 1994 by Cima and Cima, which is hereby incorporated by reference, describes tissue regeneration matrices, fabrication techniques, and

methods of regenerating tissue. In general, tissue regeneration devices can be constructed from polymers, ceramics, or from composites of ceramics and polymers. Common materials useful for constructing tissue regeneration devices are, for example, extracellular matrix proteins, especially collagens; degradable polyesters, such as polylactic acid, polyglycolic acid, co-polymers of polylactic acid and polyglycolic acid, and polycapralactone; polyhydroxybutyrate; polyanhydrides; polyphosphazenes; bone powder; natural polysaccharides, such as hyaluronic acid, starch, and alginate; hydroxyapatite; polyurethanes; and other degradable polymers described by Engleberg and Kohn, *Biomaterials* **12**: 292-304 (1991). All of these known compositions can be modified by tethering growth effector molecules to the substrate.

Growth effector molecule tethered compositions for *in vivo* use can be in the form of polymeric, attachment molecule-coated sutures, pins, wound dressings, fabric, and space-filling materials. Attachment substrates that promote ingrowth of dermal fibroblasts and capillaries could also be used for dermatological applications and cosmetic surgery, such as repair of wrinkles and aging skin, burn therapy, or skin reconstruction following disfiguring surgery. Substrates with tethered growth effector molecules that promote osteoblast migration could be used to fill bone defects following tumor surgery or for non-healing fractures. Substrates with tethered growth effector molecules that promote muscle cell growth and migration could be used for replacement of muscle mass, including cardiac muscle and smooth muscle, following disfiguring surgery and for patients with muscle degeneration or dysfunction. Tubular substrates with tethered growth effector molecules that promote growth, migration, and function of epithelial, endothelial and mesenchymal cells can be used for construction of artificial ducts for carrying bile, urine, gases, food, semen, cerebrospinal fluid, lymph, or blood. Sheaths formed of substrates that promote growth of fibroblasts from perichondrium, periosteum, dura mater, and nerve sheaths

may be used to recreate these structures when they are injured or lost due to surgery or cancer. In all of these embodiments, either the substrate with tethered growth effector molecules or substrate plus attached cells may be used for reconstruction *in vivo*.

Substrates for promoting tissue generation can be formed to have a desired tissue shape. As used herein, a desired tissue shape is the shape that the newly generated tissue is desired to have. For example, certain tissues may need to be sheet-like, tubular, or formed as a lobe.

Deactivation of the growth factor once appropriate tissue regeneration has occurred can be accomplished by tethering the growth factor to a support which slowly degrades. Examples of such support materials are polylactide-co-glycolide and crosslinked hyaluronic acid or collagen. Properly shaped substrate with tethered growth effector molecules can be applied in clinical problems such as healing of skin or periodontal ligament by encouraging continued tissue growth for the life of a shaped, degradable implant.

The disclosed compositions can be administered to animals in various modes, including implantation, injection, and infusion. Known implantation techniques can be used for delivery of many different cell types to achieve different tissue structures. The tethered growth effector molecule compositions may be implanted in many different areas of the body to suit a particular application.

Drug And Toxicity Testing Using Tissue Grown *In Vitro* On Tethered Substrates

In another embodiment, cells are cultured on substrates with tethered growth effector molecules and the resulting cell cultures are used to screen compounds for effects on cell growth, cell proliferation, cell metabolism, and DNA. For example, the cultured cells can be used to screen for compounds that alter hepatocyte enzyme systems. The cultured cells can also be used to study metabolism of various compounds and the carcinogenicity or mutagenicity of compounds both before and after metabolism by the cells.

Classically, compounds have been assayed for mutagenic activity using short term tests (STT) employing bacterial cell systems or animal studies. Most animal studies are conducted using the protocol for rodents developed by the National Cancer Institute in the early 1970s and reported by Sontag *et al.*, in *U.S. Dep. Health Educ. Welfare Publ. (NIH) Carcinog. Tech. Rep. Serv.* 1: 76 (1976). Four STTs that are routinely used are the *Salmonella* mutagenesis, SAL, described by Haworth *et al.*, *Environ. Mutagen.* 5 (suppl. 1): 3 (1983) and Mortelmans *et al. Toxicol. Appl. Pharmacol.* 75: 137 (1984); chromosome aberrations in Chinese hamster ovary cells, ABS; sister chromatid exchanges in Chinese hamster ovary cells, SCE, both described by Galloway *et al. Environ. Mutagen.* 7: 1 (1985); and mouse lymphoma cell, MOLY, assays,described by Myhr *et al.*, "Evaluation of Short-Term Tests for Carcinogens: Report of the International Programme on Chemical Safety's Collaborative Study on *in vitro* Assays" vol. 5 of Progress in Mutation Research Series, pages 55-568, Ashby *et al.*, Editors (Elsevier, Amsterdam, 1985). Unfortunately, the correlation between the rodent assays and the STTs is poor, and the available STTs do not provide a method for testing compounds for toxicity or mutagenicity of normal organ-specific cells, nor the effect of metabolism on the compounds by the organ-specific cells, such as hepatocytes.

When testing the effect of potential toxins, control assays using known toxins are used for comparison. Examples of known hepatotoxins, such as acetaminophen, carbon tetrachloride, alcohol, and cell-specific viruses such as hepatitis viruses, can be used to test the suitability of the model tissue. Standard cell number or cell lysis assays, such as Lactate dehydrogenase release, can be used to measure toxicity. Numerous other toxicity and mutagenesis assays are known in the art and can be practiced using cell cultures grown on the tethered growth effector molecule substrates described herein.

The disclosed compositions can be used to grow liver cells *in vitro* and make it feasible to use *in vitro* liver cell cultures to carry out biotransformations by applying the compound of interest directly to liver cells in culture. The supernate from the liver cell cultures can then be applied to other types of cells, such as skin, lung, nerve, and bladder, to assess any derived effect of the compound of interest. An automated system which pumps culture medium through a liver cell culture and then to cultures of these other cell types can be used.

The present invention is further understood by reference to the following non-limiting.

Example 1: Enhancement of Cell Growth.

Cell Growth And Cell Growth Assessment Methods

A. *In Vitro* Hepatocyte Culture System.

Rat hepatocytes were prepared according to Cima *et al.*, *Biotechnology and Bioengineering* 38: 145-158 (1991). Briefly, rat livers were perfused with calcium-free perfusion buffer followed by perfusion buffer with $CaCl_2$ and collagenase until the livers became soft. Cells were dispersed in William's Medium E supplemented with 10 ng/mL EGF (Collaborative Research) , 20 mM pyruvate (Gibco), 5 nM dexamethasone (Sigma), 20 mU/mL insulin (Gibco), 100 U/mL Penicillin/Streptomycin (Gibco). Cells were grown in culture generally as described by Cima *et al.* (1991). Briefly, cells were plated in culture medium at a concentration of 3 X 10^4 viable cells per square centimeter of culture surface area. Following attachment, the medium was changed to remove unattached cells and then cells were maintained in medium with daily medium changes. The base culture medium for growth on tethered substrates and control substrates was William's Medium E supplemented with 0.55 g/L sodium pyruvate, 0.5 pM dexamethasone, 0.8 mg/mL insulin (bovine), 100 U/mL

Penicillin/Streptomycin, and 2 mM L-glutamine. In some cases, the medium was supplemented with EGF.

B. Quantitative Dot-Blot Assay.

Secretion rates for the proteins albumin, transferrin, fibrinogen, and fibronectin from the hepatocyte cultures were measured with a quantitative dot-blot assay. Media samples from the cultures were serially diluted and loaded in duplicate onto nitrocellulose paper with 0.1 micron pore size using a 96 well minifold apparatus (Schleicher-Schuell). Protein standards were also loaded in duplicate at decreasing levels from 300 to 10 ng/dot. The blot was then exposed to an appropriate primary antibody for the protein being quantitated. Rabbit anti-rat albumin and anti-rat transferrin were available from Cappel. Rabbit anti-rat fibrinogen was available from Sigma. The non-bound primary antibody was washed away after one hour, and the blot was exposed to donkey anti-rabbit IgG labelled with ^{125}I (Amersham) for an additional hour. The non-bound secondary antibody was washed away, and an autoradiograph of the blot was made. The dots were then punched out and bound ^{125}I measured using a gamma counter to determine the total amount of bound antibody. A calibration curve was generated by relating known amounts of standard protein to total count per minute bound. The linear portion of the standard curve was then used to quantitate the amount of protein in the unknown media samples. Secretion rates were normalized for cell number before the modulating effects of different attachment molecule densities were compared.

C. One Dimensional SDS-PAGE of Secreted Proteins.

The pattern of protein secretion from cultured hepatocytes was determined by pulse labelling cultures from 46 to 48 hours post-attachment with ^{35}S labelled methionine (ICN) in methionine free William's E media (Gibco), with or without EGF. The media was collected after the two hour labelling, and equal amounts of protein were analyzed by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Autoradiographs were prepared with XOMAT-XAR5 film.

D. DNA Synthesis Measurement.

DNA synthesis is used as a measure of potential for cellular proliferation. Hepatocytes were pulse labelled for 20 hours beginning at 48 hours post-cell attachment with bromedeoxyuridine (BrdU), and subsequently fixed as outlined above. Cells were processed for immunocytochemistry using a BrdU kit from Amersham. Briefly, nuclei were permeabilized with DNAse I during incubation with the primary antibody. Detection of the bound antibody was achieved using peroxidase conjugated antibody to mouse immunoglobulin, and polymerizing diaminodenzidine (DAB) in the presence of cobalt and nickel, giving black staining at sites of BrdU incorporation. Alternatively, hepatocytes are pulse labelled for 16 hours beginning at 48 hours post-cell attachment with ^{3}H-thymidine, and subsequently fixed in 95% ethanol/5% acetic acid fixative for several hours. The dishes or slides were coated with Kodak NTB2 autoradiography emulsion, and allowed to expose for seven days. Autoradiographic grains were developed using Kodak D-19 developer. The percentage of cells actively synthesizing DNA was quantitated by choosing 8 random areas on each dish and counting those cells with labelled nuclei versus the total number of cells. A minimum of 35 cells was counted per dish.

<u>Synthesis of growth substrate using polyethylene oxide tether</u>

A. Silyation Reaction.

Glass microscope slides were cleaned by immersion in 1:1 methanol:HCl for at least 30 minutes. They were rinsed twice in water and immersed in 1:1 water:concentrated sulfuric acid for at least 30 minutes. After another rise in water, the slides were placed in boiling water for 15 to 30 minutes. In a glove box under a nitrogen atmosphere, the freshly cleaned slides were placed in a solution of freshly mixed acidic methanol (1.0 mM acetic acid in methanol), 5.0% H_2, and 1% ETDA (N-(2-aminoethyl)(3-

aminopropyl)trimethoxysilane) for 15 minutes, and then rinsed three times in methanol. Following the final rinse the slides were baked on a 120°C oven for 5 to 10 minutes. The slides were stored in a desiccator at room temperature while awaiting polymer grafting.

B. Activation Of Polymer.

Star polyethylene oxide was dissolved in methylene chloride (10 wt%) and dried over molecular sieve at 4°C. 110 microliters dry triethylamine and 75 microliters tresyl chloride were added to the dry polymer solution for every gram of polymer. After 90 minutes the solvent was evaporated under vacuum and the polymer was redissolved in acidified methanol (0.06 M HCl in methanol) and allowed to precipitate at -20°C. To remove unreacted tresyl chloride, the polymer was reprecipitated six times. after which the solvent was evaporated and the dried activated polymer stored under nitrogen.

C. PEO Grafting And Re-activation.

Slides were grafted with star polyethylene oxide by placing a droplet of 0.1 to 10 wt% tresyl chloride activated polymer in 0.1 M phosphate buffer (pH 7.4) on each slide and allowing the reaction to proceed for 12 hours. The slides were rinsed in phosphate buffer and then in water. Slides were dried in graded ethanol solutions of, sequentially, 25%, 50%, 75%, and 100% ethanol. Then the slides were rinsed in dry acetone and finally in dry methylene chloride before reactivation. To tresyl activate the grafted star PEO, slides were immersed for 1 hour in 0.06 M tresyl chloride, 0.07 M triethylamine in methylene chloride at room temperature under a dry nitrogen atmosphere. For mock activation controls, the tresyl chloride was omitted.

D. EGF Coupling And Desorption.

^{125}I-EGF of murine origin was coupled to activated slides in 0.01 M phosphate buffer (pH 7.4) for 12 hours at room temperature. The same procedure was followed for control slides. Adsorbed EGF was desorbed by successive washes in 0.01 M phosphate buffer (pH 7.4) with 0.1 wt% bovine collagen. The amount of EGF associated with the slides was determined

using a gamma counter. The amount of EGF coupled to activated slides was determined by subtracting the amount adsorbed to the control slides from that associated with the activated slides.

Growth of cells on tethered substrate *in vitro*

Freshly isolated rat hepatocytes were seeded on tethered EGF slides and control slides, prepared as described above. The seeded slides were incubated in William's Medium E supplemented with 0.55 g/L sodium pyruvate, 0.5 pM dexamethasone, 0.8 mg/mL insulin (bovine), 100 U/mL Penicillin/Streptomycin, and 2 mM L-glutamine. Cells were labelled with bromedeoxyuridine (BrdU) as described above.

Cell seeding and DNA synthesis assays were performed on slides that had been activated with tresyl chloride and coupled with EGF, on mock activated control slides, both prepared as described above, and on control tissue-culture treated polystyrene dishes (Falcon) either with EGF added to the medium at 10 ng/mL or with EGF omitted.

The results of the DNA synthesis assay for the latter, non-tethered controls is shown in Figure 1. The presence of EGF in the medium clearly causes an increase in the number of cells synthesizing DNA. The results of the DNA synthesis assay for the tethered EGF surface and the mock activated control surface that had only adsorbed EGF is shown in Figure 2. The number of cells synthesizing DNA is clearly higher for the tethered EGF surface.

Modifications and variations of the compositions and methods of the present invention will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

We claim:

1. A composition for stimulating the growth of eukaryotic cells comprising

a biocompatible substrate,

biocompatible tethers, and

growth effector molecules,

wherein one end of each tether is covalently linked to the substrate and each growth effector molecule is covalently linked to a distal end of a tether.

2. The composition of claim 1 wherein the form of the biocompatible substrate is selected from the group consisting of netting, individual and woven fibers, sponge and shaped polymers.

3. The composition of claim 2 wherein the shape of the shaped polymer is selected from the group consisting of dishes, bottles, solid particles, hollow particles, and polymers shaped to match a desired tissue shape.

4. The composition of claim 1 wherein the biocompatible substrate is selected from the group consisting of glasses, metals and biocompatible polymers.

5. The composition of claim 4 wherein the polymer is selected from the group consisting synthetic polymers and natural polymers.

6. The composition of claim 5 wherein the polymer is selected from the group consisting of proteins, polysaccharides, extracellular matrix proteins; polyesters; polycapralactone; polyhydroxybutyrate; polyanhydrides; polyphosphazenes; polyorthoesters, polyurethanes, and combinations thereof.

7. The composition of claim 1 wherein the tether is a water soluble, biocompatible polymer.

8. The composition of claim 7 wherein the tether is selected from the group consisting of polyethylene oxide, carboxymethylcellulose, and starch.

9. The composition of claim 1 wherein the growth effector molecules are selected from the group consisting of epidermal growth factor, platelet-

derived growth factor, transforming growth factor, hepatocyte growth factor, heparin binding factor, insulin-like growth factor I or II, fibroblast growth factor, erythropoietin, nerve growth factor, bone morphogenic proteins, muscle morphogenic proteins extracellular matrix molecules, and combinations thereof.

10. The composition of claim 1 wherein the tether has a backbone length between 5 and 50,000 atoms.

11. The composition of claim 10 wherein the tether has a backbone length between 100 and 50,000 atoms.

12. The composition of claim 10 wherein the tether has a backbone length between 5 and 500 atoms.

13. A method for growing eukaryotic cells comprising

bringing into contact the cells and a composition comprising

a biocompatible substrate,

biocompatible tethers, and

growth effector molecules,

wherein one end of each tether is covalently linked to the substrate and each growth effector molecule is covalently linked to a distal end of a tether; and

maintaining the contacting cells and composition under conditions and for a time sufficient to cause the cells to grow.

14. The method of claim 13 wherein the step of bringing into contact comprises administering the composition to a patient in need of cell growth.

15. The method of claim 14 wherein the composition is administered by injection, infusion, or implantation.

16. The method of claim 15 wherein the composition is administered by implantation of the composition and wherein the substrate is shaped to match a desired tissue shape.

17. The method of claim 16 wherein the substrate is biodegradable.

18. The method of claim 13 wherein the form of the biocompatible substrate is selected from the group consisting of netting, individual and woven fibers, sponges and shaped polymers.

19. The method of claim 18 wherein the shape of the shaped polymer is selected from the group consisting of dishes, bottles, solid particles, hollow particles, and polymers shaped to match a desired tissue shape.

20. The method of claim 13 wherein the biocompatible substrate is selected from the group consisting of glasses and biocompatible polymers.

21. The method of claim 20 wherein the polymer is selected from the group consisting of synthetic polymers and natural polymers.

22. The method of claim 21 wherein the polymer is selected from the group consisting of polylactic acid, polyglycolic acid, polyanhydrides, polyorthoesters, collagen, glycosaminoglycans, polyamino acids, and combinations thereof.

23. The method of claim 13 wherein the tether is a water soluble, biocompatible polymer.

24. The method of claim 23 wherein the tether is selected from the group consisting of polyethylene oxide, carboxymethylcellulose, and starch.

25. The method of claim 13 wherein the growth effector molecules are selected from the group consisting of epidermal growth factor, platelet-derived growth factor, transforming growth factor, hepatocyte growth factor, heparin binding factor, insulin-like growth factor I or II, fibroblast growth factor, erythropoietin, nerve growth factor, bone morphogenic proteins, muscle morphogenic proteins extracellular matrix molecules, and combinations thereof.

26. The method of claim 13 wherein the tether has a backbone length between 5 and 50,000 atoms.

27. The method of claim 26 wherein the tether has a backbone length between 100 and 50,000 atoms.

28. The method of claim 13 wherein the tether has a backbone length between 5 and 500 atoms.

29. The method of claim 13 wherein the cells are selected form the group consisting of parenchymal cells and stem cells.

30. The method of claim 29 wherein the cells are hepatocytes.

31. A cell culture comprising

a biocompatible substrate,

biocompatible tethers,

growth effector molecules, and

growing cells,

wherein one end of each tether is covalently linked to the substrate and each growth effector molecule is covalently linked to a distal end of a tether, and wherein the growing cells are bound to the growth effector molecules.

32. A method of testing a compound for an effect on tissue comprising

bringing into contact the compound to be tested and a composition comprising

a biocompatible substrate,

biocompatible tethers,

growth effector molecules, and

growing cells,

wherein one end of each tether is covalently linked to the substrate and each growth effector molecule is covalently linked to a distal end of a tether, and wherein the growing cells are bound to the growth effector molecules;

incubating the compound and the composition under conditions promoting cell growth; and

observing the cells for any effect not observed in cells not brought into contact with the composition.

DNA Synthesis - EGF in the Medium

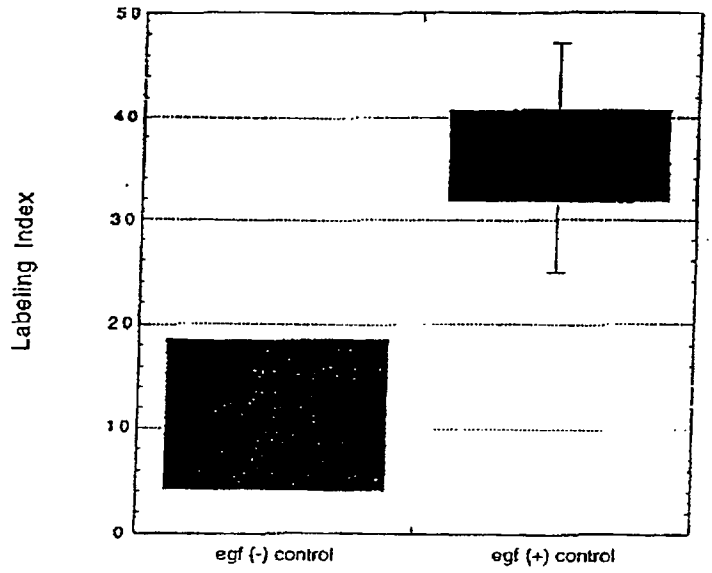

Figure 1

DNA Synthesis: Tethered EGF

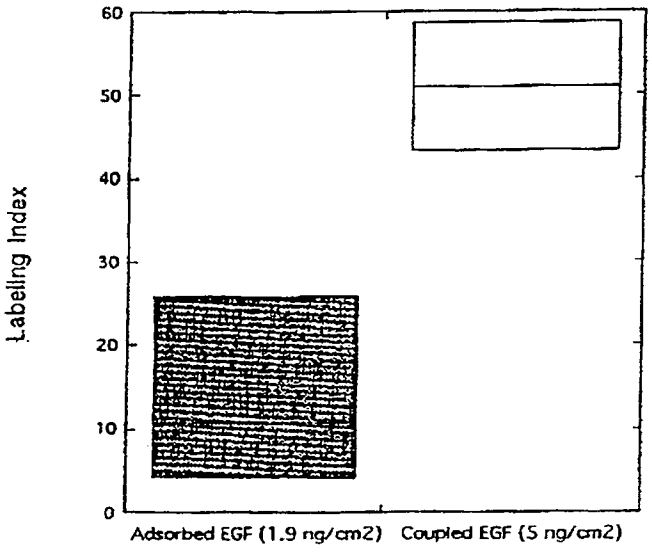

Figure 2

INTERNATIONAL SEARCH REPORT

International Application No
PC1/US 96/02851

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/00 C07K17/08 C07K17/10 C07K17/14 C07K17/12
C12Q1/00 //C07K17/06,(C12Q1/00,C12R1:91)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 531 733 (SAKAI ENEX KABUSHIKI KAISHA) 17 March 1993 see page 2, line 53 - page 5, line 31 ---	1-32
X	WO,A,89 05616 (BIO METRIC SYSTEMS INC) 29 June 1989 see page 7, line 14 - page 17, line 2 ---	1-12
A	WO,A,91 01760 (US GOVERNMENT) 21 February 1991 ---	
A	WO,A,94 28937 (ENZON INC) 22 December 1994 ---	
A	EP,A,0 205 790 (MULLER LIERHEIM KG BIOLOG LAB) 30 December 1986 ---	
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 1996	31.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Rempp, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/02851

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 9221 Derwent Publications Ltd., London, GB; Class B04, AN 92-171644 XP002009370 & JP,A,04 108 377 (UBE IND LTD) , 9 April 1992 see abstract -----	

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/ 02851

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14 - 17 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

nformation on patent family members

Inter ional Application No
PCT/US 96/02851

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		CA-A,C	2075766	13-02-93
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		DE-D-	3855238	30-05-96
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		US-A-	4828563	09-05-89
		US-A-	4789634	06-12-88

RELATED PROCEEDINGS APPENDIX

NONE